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METHODS OF PREPARATION AND PROPERTIES OF GLYCOGEN

by

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THE UNIVERSITY OF ALBERTA

METHODS OF PREPARATION AND PROPERTIES ON GLYCOGEN

A DISSERTATION

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by

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INTRODUCTION

Various methods for the preparation of glycogen are given in the literature. One of these methods, which requires the use of boiling concentrated potassium hydroxide to remove proteinous material, is thought to change or denature the naturally occuring glycogen.

These methods were studied in an attempt to determine which were the best from a consideration of the yield, ease of extraction, purity and quality of the product. In conjunction with this study a new method was developed using proteolytic enzymes, thus avoiding the use of strongly basic or acidic reagents. From a comparison of the products and several commercial samples, it was possible to draw some conclusions as to the value and effect of the different methods.

Fresh brewer's yeast, calf liver and oysters were used as the source materials for the various preparations.

LITERATURE REVIEW

a. Preparation.

Claude Bernard (8) in his physiological studies of animals at the Collège de France, established in 1850 that animals as well as vegetables were able to produce sugar. In his publication on "Nouvelle Fonction Du Foie", he reported that the process of sugar formation took place in the liver.

This was the first work on what we now know to be glycogen, although at the time when Claude Bernard commenced his studies neither the term nor the substance was known.

Claude Bernard observed that a freshly removed liver if depleted of glucose by water washing would, if left in a warm place for a few hours, produce more glucose. However, if the liver was placed in boiling water for 5 minutes it lost this property, thus indicating the presence of a heat labile substance. It is interesting to note that he thought this conversion was brought about by a kind of fermentation and that the substance was of the nature of starch. In 1855 he was successful in extracting a dry white powder from an emulsive decoction prepared from liver by precipitation with alcohol. This substance gave no tests for glucose, but could be changed by "fermentation" with brewer's yeast to glucose. He realized that this "powdered liver" was quite impure and accordingly in 1857 described a new method for preparing glycogen. It was known as the potash-alcohol method and is still used today (30). Also, at this time Bernard coined the term "glycogen" for this substance and drew a comparison between it and starch in the germinating seed. Bernard reported further that paralyzed, inactive muscles yield a glycogen which, in contrast to other samples, was colored blue with iodine. This glycogen must have been of lower molecular weight than normal, Meyer states. (26)

Hensen (30) in 1856, working independently in Germany, also isolated glycogen from liver, but Bernard because of his long and patient search was given credit for the discovery and isolation. Later, in 1859, Bernard also found that the placenta forms glycogen before the liver of the fetus begins to function.

Pasteur (32) followed Bernard in the carbohydrate field and, in 1859, he surmised that yeast contained a carbohydrate reserve substance, not necessarily glycogen. In 1882 Errera (15) substantiated Pasteur's claim when he obtained a reddish brown coloration in yeast cells treated with iodine. His efforts to separate the carbohydrate were successful.

The next examination of yeast glycogen was reported by Cremer (14) in 1894 who prepared a neutral white powder with all the characteristics of glycogen. Clautriou (12) in 1895 left a more detailed account of his studies on yeast glycogen than did Cremer. He endeavoured to increase the yield of glycogen by feeding the yeast with a 12% cane sugar solution. The resulting product had a low molecular weight.

During the period that these men had been investigating yeast glycogen, Pflüger (34) had been studying methods for estimating glycogen in tissue. He used the potassium hydroyide method of extraction, and then hydrolysed the polysaccharide with acid to glucose. He suggested the addition of a small amount of sodium chloride to aid in precipitating glycogen from the solution after the addition of an equal volume of alcohol. Pflüger used 30% potassium hydroxide solution as his modivication of the method developed by Bernard.

In 1902 a complete report dealing with the properties of glycogen was presented by Harden and Young (20) outlining their successful extraction of yeast with potassium hydroxide and alcohol. They found much mucilaginous material interfering with the precipitation and recovered a very viscous and sticky glycogen precipitate which required considerable purification.

They compared the composition, ash, specific rotation, opalescence, reaction with iodine and acid hydrolysis of yeast, oyster and rabbit liver glycogen and concluded that; "No well-marked difference exists between these 3 glycogens". However it is interesting to note that they found differences in opalescence and coloration with iodine among the various glycogen samples from different sources. The highest opalescence was obtained from the rabbit glycogen while yeast glycogen produced the lowest opalescence. When comparing iodine coloration, the rabbit glycogen was the red while oyster glycogen was the brown. At the time of publishing these results they were pioneering in the study of "Early stages in acid hydrolysis of yeast glycogen and the action on it of diastatic enzymes".

Petree and Alsberg (33) assumed that some change occurred in glycogen prepared by using potassium hydroxide. To investigate this belief they prepared glycogen from abalone meat using boiling water extractions and then precipitating the proteins present with concentrated picric acid. Unfortunately, they gave very little information about their product except that it contained 0.047% phosphorus as phosphorus pentoxide. This, they concluded, was an indication that glycogen contained phosphoric acid.

In 1930 Sahyun and Alsberg (43) produced an improved method using trichloroacetic acid as the protein precipitant instead of picric acid. They suggested the use of ammonium acetate to hasten the precipitation of glycogen. Further, they report the "denaturation of glycogen" by dry heat at temperatures above 104°C. This so-called "denatured glycogen" was slightly brown, adhesive and less opalescent than their glycogen. Bell and Young (6) were unable to confirm these results.

A study of the solubility of glycogen by Kerly (24) in 1929 produced additional proof that potassium hydroxide is too harsh a reagent on glycogen. She reported that glycogen prepared from mussels using potassium hydroxide, reached a saturation point in water at 20°C. in 2.5 hours, whereas glycogen prepared without boiling with alkali took three to four days to reach the saturation point. Also, glycogen that she found in a warm-blooded animal, was more soluble than that from cold-blooded animals.

The most recent investigation into the problem of the value of alkali extraction was done in 1950 by Bloom, Lewis, Shumpert and Shen (9). They observed that glycogen of tissues is more completely extracted by digestion with hot alkali than by extraction with hot or cold water or with acids. They also state that it is generally agreed that the glycogen found in tissues can readily be separated into "easily extracted" and "difficultly extractable fractions" by a number of different procedures. Using normal rats they studied quantitative determinations of the "total glycogen" obtainable by hot alkali digestion of liver and muscle and of the glycogen obtained by extraction of these tissues with cold 10% trichloroacetic acid.

They assumed that the hot alkali method released all the glycogen present, therefore, the difference in the amount of glycogen determined by the 2 methods may be taken as the non-acid extractable fraction of tissue glycogen. They found that a constant and reproducible proportion of the total glycogen of liver and muscle of normal rats is readily extractable with cold trichloroacetic acid. This proportion differs in the 2 tissues, 85% in liver, 55% in muscle from well fed rats.

During a fast the readily extractable fraction of the liver glycogen (trichloroacetic acid-glycogen) disappears much more rapidly than the difficultly extractable fraction.

The acid-extractable glycogen was quickly restored by feeding glucose to the rats. The acid insoluble fraction underwent relatively minor changes in fasting and feeding. Decrease in muscle glycogen following epinephrine injection was shown to occur almost wholly in the acid extractable glycogen in muscle.

From this study it seems evident that the potassium hydroxide method is better than the trichloroacetic method because it extracts more glycogen.

Meyer (26) in 1943, when summing up the problem of the potassium hydroxide effect on glycogen, stated, "In the treatment with strong potassium hydroxide, decomposition cannot be prevented unless oxygen is rigorously excluded". Accordingly, commercial glycogen has a considerably lower molecular weight than glycogen preparations which have been made with this precaution".

Other methods for the preparation of glycogen are outlined below but because of the time and laborious procedures involved are seldom used.

Stockhausen and Sibereisen (48) prepared glycogen from yeast applying a pressure of 350 atmospheres to a mixture of yeast and quartz sand. The resulting liquid which contained glycogen, mannans and proteins, was treated with Fehling's solution to remove mannans and then electrodialysed. Finally alcohol was used to precipitate the glycogen and remaining proteins. Meyer and Jeanloz (25) employed methyl alcohol, picric acid and electrodialysis to prepare pure glycogen.

Precipitation of glycogen from the mother liquor has received considerable attention in the past, but the use of ethyl alcohol as described by Bernard in reported to be the best. The attempts to improve or verify the ethanol method of precipitation by various workers is reviewed in the following paragraphs.

Sahyun (39) in 1930 attempted to improve Pflüger's laborious method for the determination of glycogen present in small amounts of tissue and found the use of activated charcoal for adsorption of glycogen remaining in the alcoholic solution quite successful. No interference with the acid hydrolysis of glycogen was noted because of this treatment.

Bancroft and Fry (4) in 1932 studied the hydrolysis of glycogen adsorbed on charcoal using hydrochloric acid, and pancreatic amylase. They stated that glycogen is strongly adsorbed by charcoal and this adsorbed glycogen is not appreciably hydrolyzed by hydrochloric acid or amylase.

Study of the variations of the 3 factors: time, temperature, and the concentration of 95% alcohol on precipitation of glycogen, was reported by Good, Kramer and Somogyi (47). They found that a high concentration of alcohol decreased the glycogen yield, while a 50% concentration allowed precipitation of glycogen, but not of dextrins. Dextrins, if present, are removed with a 65% to 75% concentration. In general, they agreed with Cori (13) who reported that two volumes of alcohol was adequate.

b. Identification and Properties.

To determine the amount of glycogen in a sample, once it has been prepared, requires hydrolysis to glucose or maltose. In early days this was accomplished by heating in 2.2 N hydrochloric acid (Pflüger) and then

neutralizing after 2 hours with 2 N sodium hydroxide. Sahyun (39) in 1930 introduced the use of sulphuric acid to eliminate sodium chloride which some workers claimed interfered with the Folin Wu sugar test then commonly used. Later, in 1933, Sahyun (40) reduced the time of hydrolysis to 20 minutes by using 5 N sulphuric acid at boiling water temperature.

The first extensive study of various animal amylases as hydrolytic agents was carried out by Barbour (5) in 1929. His findings were as follows:

(1) Trisaccharide is the sole product of fresh muscle enzyme action on glycogen.

(2) The digestion of glycogen by salivary and pancreatic amylases follows a different course producing glucose and isomaltose but no trisaccharide.

(3) At equilibrium, generally reached after 15 hours, 58% hydrolysis had occurred with muscle glycogenase.

The use of Taka-diastase as a hydrolytic agent was reported on in 1931 by Sahyun and Alsberg (42). Taka-diastase is less efficient than the muscle extract of Barbour requiring 24 hours to reach equilibrium and causing 52% hydrolysis of the glycogen.

Various color determination methods are listed but they are chiefly qualitative and used in micro-analysis of tissue. Best (17, 25) used carmine and obtained a red coloration in the presence of glycogen. Seifter and Dayton (17) and Sørensen and Hangoord (17) used anthrone and methyl resorcinol respectively to get a coloration with the glucose resulting from sulphuric acid hydrolysis of the glycogen in small amounts of tissue.

For accurate determination of the amount of glycogen, acid or enzymatic hydrolysis followed by a quantitative test for reducing sugar is required. The methods of Shaffer and Hartman, Folin Wu, and Somogyi are the most commonly used for the determination of glucose or maltose.

The properties of glycogen are as follows:

i. Physical.

a. A white powder.

b. Amorphous

c. Soluble in cold water with the formation of a stable opalescent solution.

d. Insoluble in a solution of alcohol exceeding 45% by volume.

e. Has an optical rotation of approximately: = $[\alpha]_D^{20^\circ}$ + 197°

f. Glycogen does not become insoluble in water after having been frozen and thawed, and in this it differs from amylopectin.

ii. Chemical.

a. Production of a brown to violet color with iodine.

b. Non-reducing.

c. Supposed to be alkali resistant.

d. Susceptible to acid hydrolysis yielding glucose.

e. α Amylase hydrolyses it to glucose, maltose and some low polymers.

β Amylase hydrolyses only 50% of it to maltose with the remaining 50% being limit dextrin.

f. In the presence of inorganic phosphates, phosphorylase obtained from yeast, muscle or liver catalyzes the production of glucose-I-phosphate.

c. Structure.

Two possible structures are proposed today for glycogen. They are the random branching molecule proposed by Meyer (25) and the laminated molecule proposed by Haworth (22) as shown in Figures 1 and 2.

Carter and Record (10) in 1936 suggested after much study that a molecule of glycogen may consist of 3000 - 5000 glucose units.

Two very useful procedures known as methylation and end group analysis have been of great assistance in establishing the molecular structure of glycogen.

Methylation and end group assay of glycogen, introduced by Haworth, Hirst and Webb (22) in 1929 increased the knowledge of the molecular structure, and further study by them (23) in 1937 established that the chains consisted of 12 or 18 glucose units, depending upon the source.

If methylated glycogen is hydrolyzed the principal product is 2:3:6 trimethyl glucose, but 5.5% of the glucose residues are obtained as 2:3:4:6 tetramethylglucose and about an equal amount as 2:3 dimethyl-glucose. Thus, from Haworth's work, it is shown that the glucose units are joined by α-1,4 linkages and that branching occurs through the α-1,6 linkages.

Meyer and Fuld (35) added still more to the knowledge of the constitution of glycogen with their amylase hydrolysis studies. Glycogen from mussels has an end group content of 9% (one terminal glucose unit to each II glucose units), loses 47% of its molecular weight (as maltose)

on degradation by pure β amylase, thus about five terminal glucose units are severed. All end groups appear again in the dextrin left behind; it contains 18% of end groups, i.e., one to each 5.5 glucose units. From this it may be concluded that the outermost branches of the glycogen molecule which are exposed to attack by the enzyme have an average composition of 6 to 7 glucose units in α 1,4-glucosidal linkage, of which 5.5 are removable by β amylase while 1 to 2 remain at the junctions and furnish the end groups of grenzdextrin. Between these glucose units whose number 6 position is occupied by a branch there can be only very short chains averaging 3 glucose residues with free hydroxyls at position 2, 3 and 6.

Thus it is generally believed that the exterior branches consist of 6-7 glucose units and the inner parts, between branch points, of 3 glucose units on the average. Multiple branching is possible and the larger glycogen molecules may consist of many thousands of glucose units. With such a structure, an arrangement of parallel layers of long chains into crystalline aggregates is impossible, thus explaining why glycogen is always amorphous, whereas starch is crystalline. Glycogen may have as many as 2000 branches, containing 12-18 glucose units, which build a compact molecule that is nearly spherical in shape.

The isolation of an 18 unit glycogen from rabbit liver was reported by Haworth, Hirst and Isherwood (23). Bacon, Baldwin and Bell (3) found that glycogen with a chain length of 12 glucose units was synthesized by fasting rabbits to which glucose or fructose was administered, while sucrose led to the formation of an 18 unit liver-glycogen.

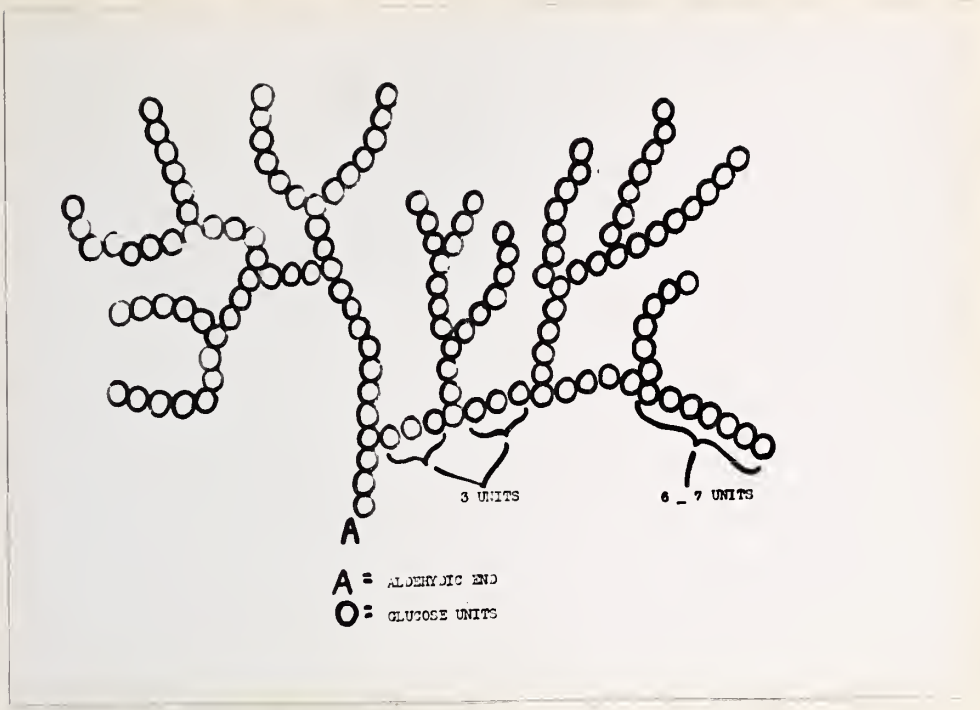

Figure 1. Diagrammatic representation of the ramified structure of glycogen (after Meyer).

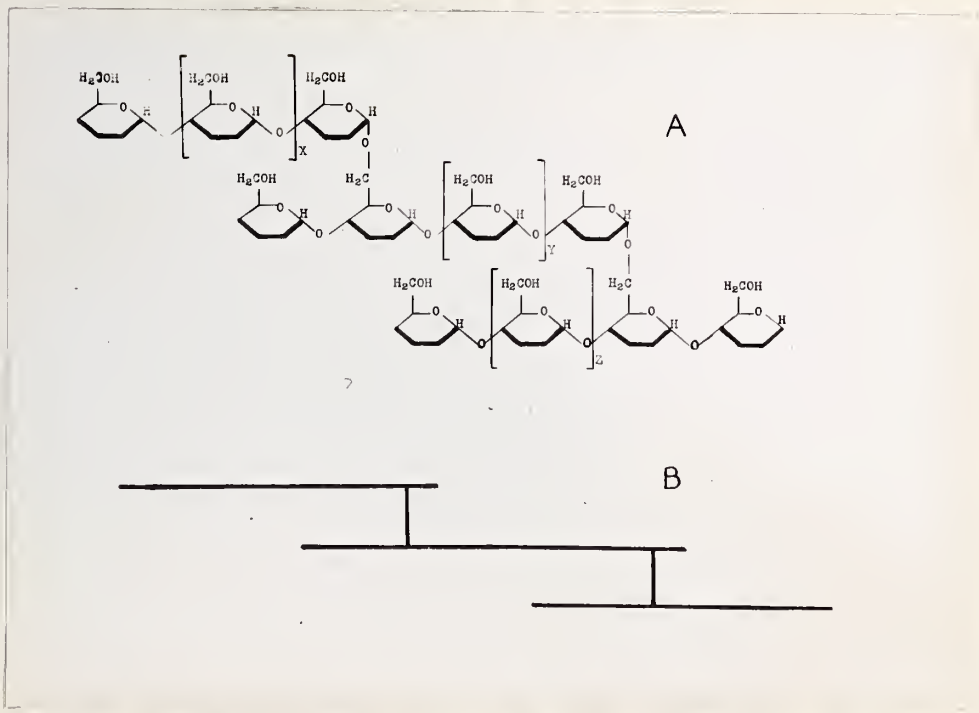

Figure 2. Formula and diagrammatic representation of the structure of glycogen (after Haworth).

Halsall, Hirst and Jones (19) in 1947 confirmed the presence of an 18 unit rabbit liver-glycogen using the periodate oxidation method instead of the lengthy methylation procedure.

Schlamowitz (45) in 1950 carried out some work on the branching characteristics of rabbit liver-glycogen to see if he could verify Bell's finding (7) but in none of the forty-six rabbits studied under different experimental conditions, was the more highly branched " - 12 unit glycogen obtained. The reported normal "end group assay value" is about 11 to 12, while he found a value of 19 which is considered to represent the abnormal glycogen.

Bell (7) obtained an "end group assay value" of 12 after the administration of glucose and fructose to rabbits while Schlamowitz obtained values ranging from 20 to 23.

Osmometric measurements have revealed that glycogen has molecular weights in the range from 2.7×10^5 to 3.5×10^6. Commercial glycogen preparations are not homogeneous, but contain molecules of lower (about 40,000) and others of very high molecular weight (2,000,000). Photographs of glycogen made with the electron microscope show that the particles have a diameter of 5-15 μ (35)

d. Occurrence and Function.

1. In the living animal organisms glycogen is found predominantly in the liver and muscle tissue, although it is present in all tissue to a lesser degree. It is also found in microorganisms such as yeast, bacteria and certain algae.

2. The presence of glycogen or glycogen-like compounds in plants was demonstrated in 1939, when it was extracted from Zea mays (sweet corn) by Morris and Morris (28). The occurrence of glycogen in sweet corn was confirmed by Hassid and McCready (21) in 1949 who further stated that the glycogen had a chain lenght of 12 glucose units. Glycogen is not morphologically differentiated like starch which occurs as discrete granules in plants, but it is distributed throughout the protoplasm.

In embryos and diabetic subjects, only, is glycogen found in the form of granules or flakes of varying size (1-10μ) in liver and muscle cells, both in the nucleus and in the cytoplasm (27). Glycogen is present in the living organism in a dynamic state and is readily formed from glucose, other sugars, sugar alcohols, certain amino acids and various organic substances. It is readily hydrolysed and synthesized by the animal body.

The distribution of glycogen in the body is quite variable even among animals of the same species which are fed on the same diets (41). The results of 7 experiments by Schondorff, in which hogs were fed for the production of as much glycogen as possible, revealed that 38% of the glycogen was found in the liver, 44% in the muscles, 9% in the bones, and the remaining 9% in the other tissues of the body. It is well known, also, that some species store glycogen in their muscles to a greater extent than others. The storage of glycogen in the body is prompted by rest as well as by liberal feeding and stored glycogen is used up rapidly during muscular work.

Various authors claim that glycogen occurs in the organism partly in a water soluble and partly in a water insoluble form (26).

There are two theories proposed for this insolubility:

1. That the insoluble portion is combined with insoluble albuminous bodies. Extracts of organs which contain glycogen and have not been freed of albumin are not broken down by the α -amylase or by β -amylase. When the albumin has been precipitated then the glycogen is attacked. The kind of union is not understood. Willstätter and Rohdewald (25) found that the better fed animals were richer in glycogen of the soluble form.

2. Meyer (26) reported that the insoluble glycogen is a high polymeric fraction which is insoluble in water and soluble in alkali. The lower polymeric fraction is soluble in water. Thus, it appears that animals that are well fed contain a large amount of the lower polymeric glycogen.

Rozenfeld and Panchenko (38) in 1950 investigated the participation of the aldehyde group in the glycogen molecule in the formation of the glycogen-protein complex, and found evidence to support the idea that the protein-glycogen comples forms by way of the aldehyde group.

Young, Evans and Tsai (49) after a thorough study of the liver of a cat report these interesting results:

a. The distribution of glycogen in the different lobes of the liver of the fasting cat is sufficiently uniform to enable comparisons between different lobes to be made.

b. Administration of amytal steadily reduces liver glycogen to approximately one-half of the original amount in 2 hours.

With inhalation of ether the liver glycogen is lowered in one hour to 40% of the original amount present.

Function.

The main physiological function of glycogen in the living organism is that of a reserve carbohydrate. Various other functions are known, but they are subordinate to this main one.

EXPERIMENTAL

1. METHODS OF PREPARATION OF GLYCOGEN.

As a result of the controversy over the effect of potassium hydroxide on glycogen, the trichloroacetic acid and picric acid methods were developed. In this way it was consdiered possible to ascertain whether different methods produced different products. However, these methods all require the use of reagents which produce pH's that are quite distant from the environmental pH of glycogen.

a. Enzymatic Preparation.

i. Fresh brewer's yeast

A method using enzymes with optimum pH's near neutrality, was devised. This method was analogous to a procedure outlined in "Advances in Protein Chemistry" (2) in which carbohydrates were removed from protein by the use of enzymes. Various procedures used for the extraction of glycogen, obtained from a review of the literature, were incorporated into the enzymatic method.

In preliminary experiments using trypsin to remove protein from glycogen many problems were encountered. Subsequent experiments were more successful and many of the difficulties at first encountered were overcome. As a result at the preliminary work it was deemed advisable to check daily the pH of the suspension of yeast extract and yeast protein with trypsin in order to test for enzyme activity. A further amount of trypsin was required at the end of the second day. Precipitates obtained from the solution by the addition of 95% ethanol, for their glycogen content as outlined in the section dealing with the examination of the glycogen samples. The precipitates were not pure but, the experience gained made possible the improvement of the procedure.

When the low glycogen content of the precipitate resulting from preparation 2 was considered, it was decided to use 2 enzymes in successive treatments. In this way trypsin would hydrolyze the protein to proteoses and peptones and a second enzyme would further the breakdown to the peptides and amino acids. Papain was selected as the enzyme suitable for this breakdown.

The complete method is outlined in detail below and is only subject to minor modifications if different enzymes or source materials are employed.

1. A sample of brewer's yeast was filtered through a Buchner funnel. A yeast cake weighing 600 grams was obtained.

2. Meanwhile, 2 litres of water in a 6 litre Erlenmeyer flask, was buffered at pH 6.5 with 100 ml. of Sørensen buffer M/15 (50). The solution was then heated to near boiling on a hot plate.

3. The yeast cake was added piece by piece to the hot water with regular shaking of the solution. When all of the yeast cake had been added the suspension was heated to boiling and shaken, then removed from the hot plate to minimize the frothing. It was then reheated to boiling.

4. This procedure was continued for 20 minutes to ensure complete denaturation of the yeast enzymes. The suspension was cooled in cold running water for 30 minutes and then centrifuged.

It was noted previously that if the suspension was allowed to stand overnight after the denaturation procedure, the supernatant solution would darken in color. This was believed due to a carbohydrate-amino acid reaction.

5. The supernatant solution (yeast extract) was divided into 2 equal portions and each one was placed in a glass-stoppered bottle. The pH of each solution was adjusted from 6.5 to 8.0 with 2N solium hydroxide.

6. The residue was suspended in 1400 ml. of water and then the suspension was divided into equal portions of 925 ml. Each portion was placed in a glass-stoppered bottle and made up to 1000 ml. The pH of each portion was adjusted to 8.0 with 2N sodium hydroxide.

7. All 4 portions were then buffered with 25 ml. of Sørensen buffer (pH 8.0). (50)

8. Ten ml. of chloroform was added to each of the 4 bottles.

9. Fifty ml. of a 2% trypsin suspension (Nutritional Biochemicals Corporation) was added to one of the suspensions of yeast residue, the other was retained as a control. The supernatant solutions were treated similarly, one being digested, the other serving as a control.

10. Fifty ml. of water was added to the controls to equalize all of the volumes. All 4 mixtures were shaken.

11. Twenty-five ml. of toluene was added to each mixture and then they were incubated at 37°C.

12. Daily pH reading were taken and the solutions readjusted to 8.0 with 2N sodium hydroxide. The daily readings are shown in Table 1.

13. At the end of the second day the 2 mixtures containing trypsin were treated with another 25 ml. of 2% trypsin suspension. At the same time 10 ml. of toluene was added to all 4 of the mixtures. Incubation was continued for 2 more days.

14. The 4 mixtures were adjusted to a pH of 7.0 with 2N hydrochloric acid and then buffered with 25 ml. of Sørensen buffer (pH 7.0). (50)

15. The addition of 50 ml. of 2% papain suspension (Nutritional Biochemicals Corporation) was carried out as stated in procedures 9 and 10 above. Daily pH readings and adjustments were made as previously outlined.

16. Very little change in the pH was noted during the next 3 days after which time all 4 mixtures were centrifuged. The 2 supernatant solutions were concentrated on the steam bath to 150 ml.

17. After centrifugation the 2 supernatant solutions were dialysed for 2 days in cold running water. Ten ml. of chloroform and 10 ml. of toluene were added to each solution before dialysis commenced.

18. The dialysates were centrifuged and the chloroform and toluene were removed.

19. The volumes of 95% ethenol was added to each of the 4 solutions. A few drops of saturated solium chloride was added to aid in the precipitation of glycogen. The 2 solutions that had been treated with enzymes produced a second precipitate with 0.5 volumes ethanol, but the untreated solutions did not.

20. The 6 precipitates were removed by centrifugation and then redissolved in water and reprecipitated. The precipitates were then washed in absolute alcohol and ether and dried over calcium chloride for 2 days in a vacuum desiccator.

The yield from 600 grams of yeast was 6.74 grams obtained as follows:

Extract minus trypsin: 0.74 grams

Extract plus trypsin plus papain$_1$: 0.84 grams

Extract plus trypsin plus papain$_2$: 1.64 grams

Protein minus trypsin: 0.72 grams

Protein plus trypsin plus papain$_1$: 1.31 grams

Protein plus trypsin plus papain$_2$: 1.49 grams

After the first few hours of incubation it was noted that the suspension of protein with trypsin added had settled faster than the suspension of protein without trypsin.

The pH readings indicated that a high rate of reaction took place the first 2 days with a progressive decrease on the successive days. The addition of papain failed to produce any change in pH, so erepsin (Nutritional Biochemicals Corp.) was used in subsequent preparations.

The yield of 1.12% was not high but the individual yields indicate that the second precipitate was larger.

The per cent glycogen of the samples was determined using the following equation;

$$\% \text{ Glycogen} = \frac{\text{no. of ml. of 0.005N } Na_2S_2O_3 \text{ for the sample}}{8.2 \text{ (Theoretical for one mgm of pure glycogen)}}$$

The values are shown in Table II It is quite obvious that the untreated mixtures had a lower glycogen content than the treated mixtures. The first precipitation with alcohol produced a sample of lower glycogen content than did the second precipitation with alcohol.

The results from this preparation were higher than those obtained for the precipitates in preparation 2, which possibly indicated the advantage of using 2 enzymes.

TABLE 1

Daily pH readings during enzyme treatment.

Mixture	Days 1	2	3	4	5	6	7
Protein suspension with trypsin	7.0	7.6	7.7	7.9			
Protein suspension minus trypsin	7.5	7.9	7.9	7.95			
Extract with trypsin	7.8	7.8	7.8	7.9	7.0	7.0	6.9
Extract minus trypsin	8.0	7.9	7.9	8.0	7.0	7.0	7.0
Protein suspension + trypsin + papain					7.0	7.0	7.0
Extract suspension + trypsin + papain					7.0	7.0	6.9

TABLE 11

Estimation of reducing sugar after acid hydrolysis of 1 mgm. of glycogen.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

Sample	Acid Hydrolysed	Control	% Glycogen
Pure glycogen	8.2	0.0	100.
Extract minus trypsin	1.8	0.0	21.9
Extract plus trypsin plus papain(1)	0.6	0.1	7.3
Extract plus trypsin plus papain(2)	3.0	0.0	36.6
Protein minus trypsin	1.6	0.0	19.5
Protein plus trypsin plus papain (1)	4.1	0.1	50.0
Protein plus trypsin plus papain (2)	5.7	0.0	69.5

With preparation number 4 an attempt was made to increase the glycogen content of the precipitate by feeding the yeast before extraction of the glycogen. Clautriou (12) mentions this in his report. The yeast was washed with water, filtered and divided into 2 equal parts "A" and "B" of 600 grams each. "A" was treated as in preparation 3, using the enzymes trypsin and erepsin (pH 7.5). Erepsin was substituted for papain as it was considered possible that erepsin would act more extensively on the proteoses and peptones. Only the yeast protein suspensions were used in this preparation. "B" was buffered at pH 6.5 and placed in 2 litres of 5% sucrose solution, for 2 hours. After 50 minutes fermentation ceased and sedimentation occurred. "B" was then processed as outlined in preparation number 3 except that the enzymes trypsin and erepsin were used. In both "A" and "B" fractional precipitation was carried out and 3 stringy, straw-colored precipitates resulted from each. Fractions "c" of both "A" and "B" were redissolved in 50 ml. of water and dialysed for 2 days in an attempt to purify them. Fractional precipitation produced 2 fractions from each of "A-c" and "B-c".

The dried and centrifuged samples were obtained as follows. The suspensions were centrifuged after enzyme action was completed and the residues were dried, thus accounting for sample "A" dried, and "B" dried. The supernatant solutions, "A" and "B", were concentrated on a steam bath to 225 ml. and then they were centrifuged. The resulting solid materials which were dried and provided samples "A" concentrated, and "B" concentrated. From solids precipitated during the course of the preparation it was possible to ascertain any loss of glycogen.

The per cent glycogen of the samples was determined and the values are shown in Table III

Precipitates "B" dried and "B" concentrate, showed a higher glycogen content than "A" dried or "A" concentrate, but the blanks of the former indicated that free reducing sugar was present. The feeding of yeast "B" did not increase the glycogen content; in fact, the precipitates showed slighly less glycogen than the precipitates from "A". Fractionation with alcohol did not prove to be of great value since the glycogen content was approximately the same in all the precipitates. The third fractionation of each solution "A" and "B" produced the highest glycogen content. Complete precipitation of the glycogen in each solution, followed by redissolving and reprecipitation would have been just as effective.

Fractionation, as described in the above preparations, has not been reported. In all work reviewed only a single precipitation with 1-2.5 volumes of 95% alcohol is used.

The yield obtained from "B" was greater than that from "A". Precipitate "c" in both "A" and "B" was the largest and had the highest glycogen content which would indicate that the higher concentration of alcohol precipitated the most glycogen. The yield from "A" or from "B" was approximately the same as that recovered from preparation 3.

The yield resulting from "A" (600 grams) was 4.03 grams obtained as follows:

"A-a"	0.85
"A-b"	0.96
"A-c_1"	0.98
"A-c_2"	1.24

The yield resulting from "B" (600 grams) was 4.81 grams.

"B-a"	1.09
"B-b"	0.75
"B-c_1"	0.53
"B-c_2"	2.44

Table II

Estimation of reducing sugar after acid hydrolysis of 1 mgm. of glycogen.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

Sample	Acid Hydrolysed	Control	% Glycogen
(#4 Yeast "A" and "B")			
"A" dried	2.0	0.1	24.4
"A" concentrated	1.6	0.2	19.5
"A-a"	3.4	0.1	41.4
"A-b"	3.7	0.0	45.1
"A-c_1"	4.0	0.0	48.8
"A-C_2"	4.2	0.0	51.2
"B" dried	2.7	0.7	32.9
"B" concentrated	2.3	0.6	28.0
"B-a"	3.0	0.1	36.6
"B-b"	3.0	0.1	36.6
"B-c_1"	1.7	0.0	20.7
"B-c_2"	4.0	0.0	48.8

TABLE IV

Daily pH readings during enzyme treatment.

Mixture	Days 1	2	3	4	5	6	7
Suspension of protein with trypsin "A"	7.2	7.6	7.6	7.9			
Suspension of protein with trypsin "B".	7.1	7.6	7.7	7.9			
Suspension of protein with trypsin and erepsin "A"					7.4	7.5	7.5
Suspension of protein with trypsin and erepsin "B"					7.4	7.4	7.5

The wall of the yeast cell has a large percentage of hemicellulose which is not attacked by the proteolytic enzymes, thus a substantial wall still remains to keep the majority of the glycogen bound in the cell. The theory that hemicellulase treatment of a yeast protein suspension followed by tryptic action might release more glycogen was tested without success, as outlined below,

Studies of hemicellulase (Nutritional Bichemicals Corp.) action on commercial glycogen using the reducing power test were made and the results are shown in Table V. The values presented in this table indicate that this sample of hemicellulase had an amylase action.

TABLE V

Estimation of reducing sugar for glycogen solutions containing hemicellulase.

Values are reported as (ml. of 0.005N $Na_2S_2O_3$)

Sample		Mgm Glucose
Glycogen plus hemicellulase (1)	12.1	1.63
Glycogen plus hemicellulase (2)	11.9	1.60
Hemicellulase plus water	0.0	

Preparation number 5, using yeast, was treated the same as preparation number 3. The yeast cake was divided into 2 equal parts, A and B, which were suspended in water. The yeast extract was treated with trypsin and erepsin as in previous experiments. "A" was adjusted to a pH of 5.0 and "B" to a pH of 6.5 since no information as to the optimum ph of hemicellulase was available.

Aliquots were taken every 24 hours for 2 days during the hemicellulase treatment of the yeast suspension and were subjected to the reducing sugar test. The results are shown in Table VI.

TABLE VI

Estimation of reducing sugar for yeast suspensions containing hemicellulase.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

Sample			Mgm. Glucose/5 ml of mixture
Yeast Suspension plus hemicellulase			
0 hrs.	A	0.5	0.07
	B	0.8	0.11
24 hrs.	A	12.7	1.71
	B	12.2	1.65
48 hrs.	A	15.8	2.14
	B	12.2	1.65

After 5 days of hemicellulase treatment the suspensions were centrifuged and the liquid saved for glycogen extraction. This produced samples (A-s) and (B-s). Water was added to the residues and

pH's were adjusted to 8, then they were digested for 4 days with trypsin. Centrifugation, dialysis, and alcoholic precipitation were carried out and samples (A-t) and (B-t) were obtained. During the hemicellulase treatment it was noted that "B" was darker in color than "A" and that "A" settled faster than "B" after shaking. Table VII shows the glycogen content of the precipitates. The values for the precipitates from (A-s) and (B-s) are low, indicating a low glycogen content, while the values obtained after tryptic digestion are appreciably higher for the precipitates.

Treatment of extract "C" gave a single sample of glycogen which showed a purity equal to A-t(2) and B-t(2).

From the values in Table VI a pH of 5.0 appeared better than a pH of 6.5 for hemicellase action. Correspondence with the Nutritional Biochemicals Corporation, who supplied the hemicellulase, gave some indication of the method of preparation, but offered little information as to further purification. Further use of hemicellulase was discontinued. Yields were not considered in this preparation.

TABLE VII

Estimation of reducing sugar after acid hydrolysis of 1 mgm of glycogen.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

Glycogen resulting from hemicellulase and trypsin action

Sample	Acid	Water	%Glycogen
A-s_1	3.1	0.0	37.8
A-s_2	2.8	0.0	34.1
B-s_1	3.1	0.0	37.8
B-s_2	2.9	0.0	35.4
A-t_1	3.1	0.0	37.8
A-t_2	4.7	0.0	57.3
B-t_1	3.5	0.1	42.6
B-t_2	4.6	0.0	56.1
C_1	4.7	0.0	57.3

ii. Calf liver

Fresh calf liver was used as the source material for enzymatic preparation 6. A calf was fed a normal diet 4 hours before slaughtering, and the whole liver was then removed immediately after slaughtering, then placed in boiling water one-half hour later to destroy the enzymes present. The enzymes used in preparation 6 were papain followed by erepsin. The procedure was similar to that of number 3 except that 200 grams of liver pulp was used. The Waring Blendor was used to chop the liver finely. After the enzyme treatment was terminated the supernatant solution was heated to 85°C in an attempt to remove any soluble protein. The resulting solid was concentrated by vacuum

distillation but frothing caused much interference. Fractional precipitation was also carried out in this preparation. The precipitates were brown in color, which is not characteristic of pure glycogen. The per cent glycogen was determined and the values shown in Table VIII indicate that very impure precipitates were obtained. The yield was not high in this preparation.

The yield resulting from 200 grams of liver was 2.97 grams obtained as follows:

Precipitates: "a": 0.34 grams.
"b": 0.17 grams.
"c": 0.39 grams.
"d": 0.23 grams.
"e": 1.54 grams.
"f": 0.34 grams.

TABLE VIII

Estimation of reducing sugar after acid hydrolysis of 1 mgm glycogen.

Values are reported as (ml. of 0.005N $Na_2S_2O_3$)

Sample	Acid Hydrolysed	Control	% Glycogen
"a"	1.2	0.1	14.9
"b"	1.1	0.0	13.4
"c"	1.3	0.0	15.9
"d"	1.1	0.0	13.4
"e"	1.8	0.1	21.9
"f"	2.5	0.1	30.5

Glycogen was again prepared from calf liver (preparation seven) using the enzymes trypsin and erepain and the same procedure as in preparation number 6 was followed. Heat was not used to precipitate any protein present. Each precipitate was very viscous and dark brown in color and indicated a very poor quality of glycogen. The per cent glycogen values are shown in Table IX and are higher than those for preparation 6. The enzyme combination may have been better in this preparation. The protein did not precipitate. The increase in volume during dialysis indicated water going in instead of internal components coming out, therefore, these components must have been of high molecular weight (large molecule) and were probably polypeptide in nature. This also indicated that there must have been too much protein in proportion to the enzymes. The yield from 500 grams was 3.02 grams.

TABLE IX

Estimation of reducing sugar after acid hydrolysis of 1 mgm of glycogen.

Values are reported as (ml. of 0.005N $Na_2S_2O_3$)

Sample	Acid Hydroysed	Control	% Glycogen
(#7 - Liver)			
1	3.0	0.1	36.6
2	3.3	0.0	40.2
3	1.3	0.0	15.9
4	2.8	0.1	34.1
5	3.3	0.0	40.2
6	3.2	0.1	39.0

iii. Oysters

Oysters were used as the source material in preparation 8, in which the enzyme combination of trypsin and erepsin was used. The oyster pulp was extracted 3 times in boiling water before the extract was treated as outlined in preparation 3. The oysters were shelled previous to shipment in ice, from the Atlantic coast.

Two precipitates were recovered from the extract. They were redissolved and precipitated with 95% alcohol. Five precipitates were obtained.

The yield from 300 grams was 4.08 grams.

1a: 0.42 grams

2a: 1.13 grams.

2b: 0.93 grams.

2c: 0.96 grams.

2d: 0.64 grams.

The glycogen content of the precipitates is shown in Table X. The blanks did not indicate the presence of free reducing material. All fractions of precipitate 2, except 2c, showed values greater than the enzymatic preparation was moderately effective on oysters, possibly because of the higher degree of hydration of the protoplasm and absence of a rigid wall. Fraction 1a was not very high in glycogen content and thus it was indicated that fractionation was not necessary. All samples were soluble in water and gave opalescent solutions. The yield was good when compared with the other yields.

TABLE X

Estimation of reducing sugar after acid hydrolysis of 1 mgm. of glycogen.

Values are reported as (ml. of 0.005N $Na_2S_2O_3$)

Sample	Acid Hydrolysed	Control	% Glycogen
(Oyster-enzyme)			
1a	2.1	0.0	25.6
2a	5.4	0.0	65.9
2b	5.1	0.0	62.1
2c	4.7	0.1	57.3
2d	5.2	0.0	63.4

Two preparations, 9 and 10, employing enzymes were carried out using oysters and fresh calf liver extract. The procedures were identical to that for preparation 8 except that one precipitation only was made from each solution, employing 2.5 volumes of alcohol. This procedure was carried out 5 times after which the sample was washed in absolute alcohol, ether, and then dried in vacuo over calcium chloride. The sample was then powered, weighed, and stored in a desiccator. The glycogen content of these samples is given in Table XI.

The yield for each sample was as follows:

Preparations 9: 0.26 grams.

10: 0.53 grams.

Redigestion of a glycogen sample prepared from yeast, using enzymes, was attempted to determine whether a purer sample could be obtained. The glycogen sample used was from the first alcoholic precipitation in preparation number 3. One gram of glycogen was

dissolved in 50 ml. water and treated with trypsin (0.1 grams) for 4 days. The pH was adjusted daily. The enzyme action was rapid the first day but slower on successive days. The sample was dialysed for 2 days against running water (T= 17°C) after the tryptic digestion, then 4 volumes of 95% alcohol plus 7 drops of sodium chloride were added to precipitate the glycogen. The precipitate was dissolved in water, and reprecipitated twice, washed with absolute alcohol and ether and dried over calcium chloride in vacuo. The yield of light brown material was 0.24 grams, indicating that much of the original material must have been protein in nature or, if not, a high loss occurred during treatment. The per cent glycogen of the retreated sample was 54.9 while that of the original sample was 50.0.

TABLE XI

Estimation of reducing sugar after acid hydrolysis of 1 mgm of glycogen.

Values are reported as (ml. of 0.005N $Na_2S_2O_3$

Sample	Acid Hydrolysed	Control	% Glycogen
Preparation Nine (oyster) . .	5.5	0.0	67.7
Preparation Ten (calf liver extract) . .	3.0	0.0	36.5
Retreated Sample	4.5	0.0	54.9
Trypsin	0.2	0.2	

b. The Potassium Hydroxide Method.

i. Fresh brewer's yeast

The mode of preparation of commercial glycogen is not definitely known although it is reported (26) that the strong potassium hydroxide method is used. Two samples of glycogen were prepared from brewer's yeast using the general procedure (1,6,). The same sample of yeast was

used as in the enzymatic preparation number 3. The procedure was as follows:

1. The yeast was filtered as described for enzymatic preparation 3 and a portion of the yeast cake was used for the potassium hydroxide preparation.

2. Six hundred grams of yeast cake was suspended in 1200 ml. water.

3. Six hundred ml. of hot potassium hydroxide was added to the suspension, producing a 20% potassium hydroxide solution of pH 12.4. The suspension was heated for 90 minutes on the water bath.

4. The solid material soon dissolved and a dark brown, clear solution resulted.

5. The solution was removed from the water bath and centrifuged while warm. (55-60°C)

6. An equal volume of 95% ethyl alcohol was added to the warm solution causing a heavy, cream-colored precipitate.

7. The precipitate was removed by centrifugation after 20 minutes and redissolved in 500 ml. water. An equal volume of alcohol was again added.

8. The suspension was again contrifuged and the precipitate was washed with 900 ml. of a mixture of one part 15% potassium hydroxide and 2 parts 95% alcohol.

9. The suspension was recentrifuged and the precipitate dissolved in 500 ml. water. The resulting solution was divided into 2 equal parts, "A" and "B".

10. Glycogen was precipitated with an equal volume of 95% alcohol from part "A". The sample was washed with absolute alcohol, ether, and then dried in vacuo over calcium chloride for 2 days.

Yield: 2.98 grams.

As yeast contains mannans and galactans, it was considered desirable to prepare a sample not containing them so that a comparison could be made. Part "B" from the above procedure "A" was prepared in this manner as follows:

1. Part "B" was neutralized with IN sulphuric acid and the albuminous residue was removed by centrifugation.

2. To the supernatant liquid an excess of Fehling's solution (30 ml. at pH 12.8) was added, and the entire solution was allowed to stand for 15 hours. Fehling's solution was added to remove mannans (26). No precipitate occurred with the addition of the Fehling's solution but after 15 hours a blue precipitate had settled out.

3. The supernatant liquid was neutralized with IN sulphuric acid before being dialysed.

4. Dialysis was continued for 3 days in an attempt to remove the blue color. The glycogen was then precipitated by the addition of one volume of 95% alcohol and a few drops of sodium chloride.

5. The sample was washed with 95% alcohol, absolute alcohol, and ether, and then dried as previously outlined.

Yield: 1.26 grams.

The blue coloration persisted indicating that continued purification would be required.

A comparison of the 2 glycogen preparations using the reducing sugar test is shown in Table XII. "B" had a much greater content than "A".

A test was made to determine the effect of Fehling's solution on a pure glycogen solution. A 0.5% solution of commercial glycogen was treated with an excess of Fehling's solution of pH 12.8 and allowed to stand for 15 hours. No precipitate was found, therefore this, and the reducing power test, indicated that the Fehling's solution treatment was useful. No great difficulty arose in these preparations which is contrary to the report of Harden and Young (20) If a larger amount of yeast solid is used in proportion to potassium hydroxide solution than outlined in this preparation, the precipitation is unsatisfactory. This may account for Harden and Young's difficulties with extraction.

Yeast was again used as a source material for potassium hydroxide preparation (2). One variation was used from the former preparation in that half of the solution "B" was neutralized with acetic acid after potassium hydroxide treatment. A larger proportion of yeast material to potassium hydroxide was used than the one to 3 ratio previously found to be satisfactory.

Much difficulty, due to interfering mucilaginous matter, was encountered throughout the whole preparation in both the neutralized and unneutralized portions. Table XII shows per cent glycogen values. From these it appeared that the unneutralized portion yielded precipitates of slighly higher glycogen content than did the neutralized portion. The values for these precipitates are almost identical with that for potassium hydroxide sample "A" of preparation one, which would indicate that although all 3 methods of preparation varied one with the other the glycogen values obtained, were approximately the same.

ii. Calf liver

Two hundred grams of the fresh calf liver used in the enzymatic preparation number 6 was subjected to the potassium hydroxide method. The procedure was as outlined previously. Within half an hour after the reaction commenced the solution was free of solid material. The precipitate obtained was purified several times after which a white sample resulted.

Yield 3.71 grams.

This preparation, which required 2 days, provided no special difficulty and precipitation with alcohol from the alkaline medium did not present a problem as Pflüger's method (42) did for some workers. The per cent glycogen of the sample is shown in Table XII and indicates a high purity of glycogen. In this preparation the amount of solid in proportion to potassium hydroxide was kept to approximately a ratio of one to 3 as in the yeast-potassium hydroxide preparation.

Another preparation using fresh calf liver was carried out using hot concentrated (20%) potassium hydroxide. A larger proportion of liver material was used than in the previous preparation with the result that a low purity product was obtained and difficulty in extraction was encountered due to jelling of both solutions. Both the solid liver material and the extract were treated with hot concentrated (20%) potassium hydroxide and a whiter product was obtained from the extract. This indicated that an excess of protein-like material must have been present after potassium hydroxide treatment was discontinued. ~~The~~ The per cent glycogen of the precipitates shown in Table XII indicated that neither sample was pure and that the original liver extract contained less glycogen than the solid material.

Liver solid material: yield . . 1.39 grams.

Liver extract: yield . . 3.60 grams.

TABLE XII

Estimation of reducing sugar after acid hydrolysis of 1 mgm. of glycogen.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

Sample	Acid Hydrolysed	Control	% Glycogen
(Potassium hydroxide preparations.)			
1 A Yeast	5.8	0.0	70.7
1 B Yeast plus Fehling's solution. . .	6.8	0.0	82.9
2 A_1 Yeast-Alkaline	5.9	0.0	71.9
2 A_2	5.1	0.0	62.2
2 B_1 Yeast Neutralized	5.7	0.0	69.5
2 B_2	4.3	0.0	52.4
3 Calf liver solid material .	7.5	0.0	91.5
4 Calf liver solid material .	5.1	0.0	62.2
4 (Liver extract)	3.3	0.0	40.2

c. The Trichloroacetic Acid Method.

i. Calf liver

A portion of the liver that was used for the enzymatic and potassium hydroxide methods was used in the first trichloroacetic acid preparation. The reports by Bell and Young (6) and Plimmer (36) were used for reference. The procedure was as follows:

1. Two hundred grams of finely ground liver was added to 1000 ml. of boiling water and the suspension was allowed to boil for 5 minutes.

2. The suspension was centrifuged and the supernatant solution was retained.

3. The residue was boiled with 400 ml. water for 10 minutes, centrifuged, and the 2 supernatant solutions were combined. The pH of the solution was 6.5.

4. One-tenth the volume of 40% trichloroacetic acid was added to the solution (pH 1.5) which was then refrigerated for 18 hours. A cream-colored precipitate formed after the acid was added.

5. The heavy precipitate was removed by centrifugation and one volume of 95% alcohol was added to the supernatant. A white precipitate formed.

6. The white precipitate was removed by centrifugation and treated with 95% alcohol, absolute alcohol, and ether. It was then dried over calcium chloride in vacuo at room temperature for 2 days.

Yield: 1.23 grams.

The product looked quite similar to the commercial samples and was quite dense. This preparation was simpler than the potassium hydroxide preparation using liver.

A second trichloroacetic acid preparation using 500 grams of liver was made using the same procedure with one modification. Three extractions of the liver pulp for 30 minute periods were used in place of the former 2 short extractions.

A white, fluffy sample was recovered.

Yield: 1.54 grams.

ii. Oysters

Another preparation using oysters was made with trichloroacetic acid as the protein precipitant.

The pH of the oyster suspension was 6.2. The preparation was identical to the previous one, yielding a white sample.

The yield from 161 grams of oysters was 2.40 grams.

These glycogen preparations, employing trichloroacetic acid as the protein precipitant, were relatively simple compared to the other methods outlined. The yield was not high in any of the trichloroacetic acid preparations, possibly indicating that water extraction is not efficient or that acid hydrolysis of the glycogen had occurred. The per cent glycogen values in Table XIII show that the resulting samples were of fairly high glycogen content with the exception of the oyster sample, which was low.

d. The Picric Acid Method.

1. Oysters

Two methods employing picric acid are outlined in the literature. Each was followed using oysters as the source material. Petree and Alsberg (33) outline very briefly the steps in the method but no specific quantities are stated. The procedure used was as follows:

One hundred and sixty-one grams of (18 oysters) oysters were finely ground and extracted with water as outlined under the second trichloroacetic acid preparation. An excess of concentrated picric acid (1 litre) was added to 750 ml. of the extract (pH 1.0) to precipitate the protein present. Concentrated picric acid is prepared by saturating a solution of water with picric acid. The suspension was cloudy but no precipitate formed during refrigeration overnight. The addition of more concentrated picric acid failed to produce precipitation. The suspension was neutralized with 2N sodium hydroxide then concentrated in vacuo to 750 ml. This produced a dark-brown colored suspension. This suspension was centrifuged and the supernatant solution was retained. This solution produced a yellow-colored

precipitate after the addition of 2.5 volumes of 95% alcohol. Six purifications by solution and reprecipitation were necessary in order to remove the yellow coloration due to picric acid. Yield: 1.82 grams.

Picric acid was shown to be a poor protein precipitant in this preparation when compared with trichloroacetic acid.

Meyer and Jeanloz (26) describe a more complete method using picric acid and absolute methyl alcohol. The procedure was followed exactly as they reported using an oyster extract which was identical to the one mentioned in the first picric acid method. A grey colored precipitate was recovered. Six purifications were required to remove the yellow coloration from the precipitate. Yield: 2.27 grams.

As in the former preparation, the picric acid failed to precipitate proteins. The purity based on the per cent glycogen of the 2 products is lower than those for trichloroacetic acid as shown in Table XIII. The pH (O.G.) was lower for picric acid solutions than for those using trichloroacetic acid. (pH 1.5)

TABLE XIII

Estimation of reducing sugar after acid hydrolysis of 1 mgm. of glycogen

Values are reported as ml. of 0.005N $Na_2S_2O_3$.

Sample	Acid	Hydrolyzed Control	% Glycogen
(TCA preparations)			
#1.	7.1	0.0	86.6
#2.	7.5	0.0	91.5
#3. . (oysters)	6.2	0.0	75.6
(Picric Acid preparations)			
#1 Petree-Alsberg	5.6	0.0	68.3
#2 Meyer	6.5	0.0	79.3

11. EXAMINATION OF THE GLYCOGEN SAMPLES.

In order to compare the various methods of preparation eighteen glycogen samples were examined using a number of tests.

A list of the samples is given below:

1. Commercial glycogen . . . . oysters)
2. Pfanstiehl glycogen . . . . C.P.)
3. Pfanstiehl glycogen . . . . practical) Commercial preparations of glycogen.
4. General Biochemicals))
5. Nutritional Biochemicals))
6. Trichloroacetic glycogen . . . . oysters, preparation 3.
7. Trichloroacetic glycogen . . . . calf liver, preparation 2.
8. Trichloroacetic glycogen . . . . calf liver, preparation 1.
9. Potassium hydroxide glycogen . . yeast, preparation 1A.
10. Potassium hydroxide glycogen . . liver, preparation 3.
11. Potassium hydroxide glycogen . . liver, preparation 4.
12. Potassium hydroxide glycogen . . liver extract, preparation 4.
13. Enzymatic glycogen . . . yeast (protein plus trypsin plus papian, second precipitate, preparation 3.)
14. Enzymatic glycogen . . . yeast redigestion, preparation 3.
15. Enzymatic glycogen . . . oyster, preparation 9.
16. Enzymatic glycogen . . . liver extract, preparation 10.
17. Picric acid glycogen (Petree and Alsberg) . . oysters, preparation 1.
18. Picric acid glycogen (Meyer) . . . oysters, preparation 1.

The tests used were as follows:

1. Estimation of moisture.
2. Per cent purity.
3. Total nitrogen using the Micro-Kjeldahl apparatus, calculated to per cent protein.
4. Ash content.
5. Iodine coloration using the Beckman Spectrophotometer.
6. Osmotic pressure studies.
7. Viscosity.
8. Periodic Acid oxidation.
9. Cupric chloride crystallization pattern test.
10. Protein glycogen binding test.
11. Presence of iron (Fe^{+++})

All the samples were moisture free when weighed for the various tests.

The first requirement in the experimental work was a good qualitative test for glycogen. Various methods of testing for the presence of glycogen are known among which are 3 staining methods listed in Glick's "Technique of Histo- and Cyto-Chemistry" (17). These, however, are for glycogen deposited in histological preparations, yet they were tried.

The methods were studied as follows:

i. Bauer-Feulgen's Stain.

The Feulgen reagent which is straw-colored, contains 1 gram of basic fuchsin in 100 ml. water plus 2 ml. 1N hydrochloric acid and 1 gram sodium bisulphite. It is used for staining slides bearing tissue. Different amounts of the reagent were added to 0.1 per cent and 0.01 percent solutions of glycogen and to water, and the colora-

tion produced was noted. All the solutions became a red-violet in color with no visible differences among them.

ii. Best's Carmine Stain.

This solution contains 2 grams carmine, 1 gram potassium carbonate and 5 grams potassium chloride in 60 ml. water. Twenty ml. of concentrated ammonium hydroxide is added to this solution. Different amounts of this reagent were added to 0.1% and 0.01% solutions of glycogen and water with the production of a red coloration. No obvious visual difference could be seen among the solutions.

iii. Gomori's Procedure.

This method required many washings of the tissue with different reagents. The addition of alkaline silver methenamine and gold chloride produced a white suspension. It itensified in the water solution upon incubation but not with the glycogen. After 2.5 hours of heating at 45°C, the glycogen solution was dark brown, while the water solution was lighter and contained a precipitate. This method also seemed to be unsuitable for the purpose.

None of these staining methods was adapted to this study due to the unreliable results obtained.

iv. Boettinger's Method.

A colorimetric method developed by Boettinger was tested and found to be of little value. The method is outlined by Glick (17). In this method 2.5 volumes of diphenylamine is added to one volume of glycogen after which the mixture is heated for 40 minutes in boiling water and then examined in the Evelyn colorimeter. The various solutions took on a blue tinge when heated, which varied in intensity for different concentrations, the water showed a slight blue tinge.

Readings were made which showed a difference between 1.0%, 0.1% and 0.01% solutions of glycogen. The ease of oxidation of diphenylamine, especially when heated, makes the test inaccurate for quantitative work.

v. Hydrolytic-Reducing Sugar Method.

The most logical approach was to determine the amount of reducing sugar resulting after acid or enzymatic hydrolysis of a definite amount of glycogen. The Somogyi method (44) was incorporated into a procedure which was used throughout the entire study. One litre of this reagent contains the following:

28 grams of anhydrous disodium phosphate

100 mls. of normal sodium hydroxide

40 grams of Rochelle salt

8 grams of cupric sulphate (crystals)

180 grams of anhydrous sodium sulphate

"x"= ml. of normal potassium iodate

"x"=25 ml. for up to 3 mgm. glucose in a 5 ml. solution and

"x"=10 ml. for up to 1 mgm. glucose in a 5 ml. solution.

In the reagent used in this study 25 ml. potassium iodate was used. The procedure depends upon the type of hydrolysis whether acidic or enzymatic.

A. Acid Hydrolysis.

Five mg. glycogen is placed in a 10 ml. pyrex test tube to which is added 5 ml. 5N sulphuric acid. The whole is shaken and then a round stone ball is placed on top of the tube which is immediately immersed in vigorously boiling water for 20 minutes. After cooling in water for 5 minutes the contents of the tube are placed in a 25 ml. volumetric flask to which is added 5 ml. 5N sodium hydroxide to neut-

ralize the acid present. The volume is made up to 25 ml. with water and after shaking, a 5 ml. aliquot is placed in a test tube to be tested for the amount of reducing sugar present. A blank is run with the test sample using 5 ml. water instead of 5 ml. 5N sulphuric acid.

Five ml. of the neutralized solution should contain the equivalent of one mgm. hydrolysed glycogen.

Tests were made to check on the time interval and acid concentration of the aforementioned procedure. Sahyun (39) reported that 5N sulphuric acid for a 20 minute boiling period was satisfactory.

Pure glucose and a commercial glycogen were tested with 5 ml. of 5N sulphuric acid over time intervals of 10, 20, 30 and 60 minutes. Tests were also made using the same 2 materials with 5 ml. of 2N sulphuric acid and the time intervals of 10, 20, 30 and 60 minutes and 1, 2, 3 and 4 hours. Eighteen N sulphuric acid was tested using a time of 10 minutes. All neutralizations were made using 2 or 5N sodium hydroxide as required. The results of this study are shown in Table XIV.

From this it was indicated that 5 ml. of 5N sulphuric acid, if used for a 20 minute boiling period, was satisfactory for complete hydrolysis. Further, it showed that glucose under the above conditions was not altered. The other low concentration was satisfactory but the time involved was greater. Concentrated sulphuric acid was not suitable.

TABLE XIV

Estimation of reducing sugar after acid hydrolysis of 1 mgm. glycogen or glucose

Values are reported as ml. of 0.005N $Na_2S_2O_3$.

(1 mgm. glucose requires 7.40 ml. of 0.005N $Na_2S_2O_3$)
(1 mgm. glycogen requires 8.21 ml. of 0.005N $Na_2S_2O_3$)

Solution	Minutes	10	20	30	60	120	180	240
5 ml. ½N Sulphuric Acid								
Glucose		7.2	7.4	7.4	8.7			
Glycogen		7.0	7.7	7.7	9.1			
5 ml. 2N Sulphuric Acid								
Glucose		5.1	6.0	7.1	7.3	7.4	7.4	7.4
Glycogen		1.0	1.5	3.7	5.7	7.2	7.4	7.6
18N Sulphuric Acid								
Glucose		12.1						
Glycogen		11.2						

The Somogyi Method (47) was used for quantitative determinations of reducing sugar. Values were obtained for glucose using pure glucose so that conversion of the amount of sodium thiosulphate used to the amount of glucose present could be made when glycogen hydrolysis was studied. Tests made produced results which checked with those reported by Somogyi. It was found that 7.40 ml. of 0.005N sodium thiosulphate was equal to one mgm. glucose, and that 3.84 ml. was equal to one mgm. of maltose. Graph number 1 (Figure 3) shows the relationship of mgm. of glucose and mgm. of maltose to ml. 0.005N sodium thiosulphate.

B. Enzymatic Hydrolysis.

The enzymes α amylase and β amylase were used to hydrolyse the glycogen samples.

α Amylase action starts at the end of the branches in a glycogen molecule and cleaves the glucose units singly, in pairs, or in low polymers. The reducing sugars present, therefore, are not of one specific type and the measure of the amount of reduction represents glucose, maltose and low polymers of glucose. α Amylase action does not go to completion, thus a lower reducing sugar concentration is obtained than when acid hydrolysis is used.

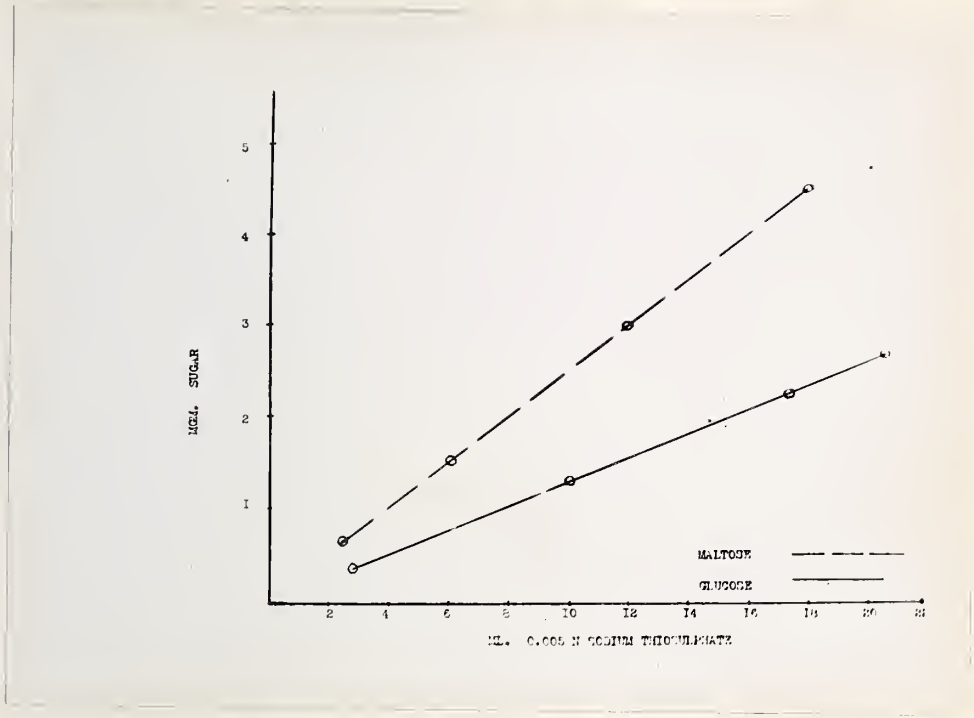

Figure 3. Relationships of mgm. of glucose and mgm. of maltose to 0.005N sodium thiosulphate.

β Amylase is more specific in its action cleaving the branches into maltose units only. β Amylase cannot hydrolyse past the branching points as α amylase can. Approximately 40% of the polysaccharide is hydrolyzed, thus a lower reducing sugar concentration if found when compared with α amylase or acid hydrolysis.

A conversion factor is required for glycogen to maltose as 1 mgm. of glycogen is equivalent to 1.055 mgm. of maltose. With this it is possible to calculate that one mgm. of glycogen when completely hydrolysed would require 4.05 ml. of 0.005N sodium thiosulphate. Forty per cent of this amount is 1.62 ml. which is the theoretical amount expected after β amylase action on one mgm. glycogen.

The hydrolysis procedure is as follows:

Twenty-six mgm. glycogen are placed in a 25 ml. graduated glass-stoppered flask and 20 ml. water is added. When the glycogen has dissolved one ml. of sodiumacetate-acidic acid buffer(o.1 m. pH 4.67)if α amylase is used or 5.8 (M/15 - McIlvaine's (50) if β amylase is used. Five ml. of a freshly prepared 0.08% amylase solution is added. The solution is well shaken, then incubated at 30°C for 24 hours. Two ml. aliquots are removed at various time intervals and the amount of reducing sugar is determined. Two ml. aliquots are used as only small amounts of reducing sugar are present in the early stages of the hydrolysis. The final value is divided by 2 to give the amount of reducing sugar resulting from one mgm. of glycogen.

The α amylase, Clarase (Eimer and Amend) was used in preference to the α amylase, Taka-diastase (Parke and Davis Co.) after the hydrolytic action of both had been tested on a one per cent solution of soluble starch. As shown in Table XV the Clarase action was superior to that of Taka-diatase.

TABLE XV

Estimation of reducing sugar after enzymatic hydrolysis of a 1 per cent starch solution

Values are reported as ml. 0.005N $Na_2S_2O_3$

Solution	20 min.	60 min.	120 min.
Starch plus Taka-diastase.	8.3	18.4	21.9
Starch plus Clarase.	16.5	21.9	22.4

The use of toluene and chloroform as preservatives was tested using a commercial glycogen sample and Clarase. The results are shown in Table XVI. After 24 hours there was no difference between the solutions but after 96 hours a marked difference was found between the solutions. This test indicated the possibility of the growth of a micro organism, and also that the different preservatives did not interfere with any of the reactions the first 24 hours.

TABLE XVI

Estimation of reducing sugar after enzymatic hydrolysis of 1 mgm. glycogen.

Values are reported as ml. 0.005N $Na_2S_2O_3$.

Solution	24 hours	96 hours
No toluene.	3.3	5.5
Toluene.	3.3	6.7
Toluene plus nitrobenzene	3.3	6.2
Toluene plus chloroform	3.3	5.5
Clarase plus toluene plus water.	0.05	0.1

1. Moisture Determination. The per cent moisture of all eighteen samples was determined as follows:

A 4 ml. glass cup was brought to constant weight by drying at 106°C in an oven. One half of a gram of glycogen was placed in the dish and the whole was weighed before and after heating at 106°C for 2 hours. The difference in weight was obtained and the per cent moisture calculated. The results are shown in the composite table. Two samples were heated for 10 hours and on further change in weight was noted. Several tests were made to check the finding of Sahyun and Alsberg (42) who stated that glycogen is degraded if heated over 104°C. The term "denatured" is used by Sahyun and Alsberg but it is incorrect.

Four commercial glycogen samples, weighing 0.5 grams each, were heated for 24 hours at 106°C after which the per cent glycogen was determined. The values were identical with the values obtained before heating, which was a most important result, but they also looked the same before and after heating. From this it was concluded that heating over 104°C does not denature glycogen.

2. Per cent Glycogen. All the samples were hydrolysed with acid and the per cent glycogen determined from the reducing sugar values obtained. Table XVII shows the results which indicated that no sample had a higher per cent purity than 95. A low purity of 36.6 per cent was found.

The values obtained for the samples after Clarase hydrolysis were well related to the purity of the samples as determined by acid hydrolysis. In 2 cases Clarase hydrolysis was repeated and continued for 48 hours. No increase in the amount of reducing sugar was noted during

the extended period of hydrolysis. The curves obtained for typical samples representing each method of preparation are shown on Graph number 2 (Figure 4).

Three representative samples were hydrolysed with β amylase for 48 hours. No further hydrolysis was found after 24 hours. With β amylase approximately 40% of the sample should have been hydrolysed.

The percent hydrolysis was calculated for these 3 samples and, as shown in Table XIX and in Graph number 3 (Figure 5), only in one case did it come to the value of 40% hydrolysis.

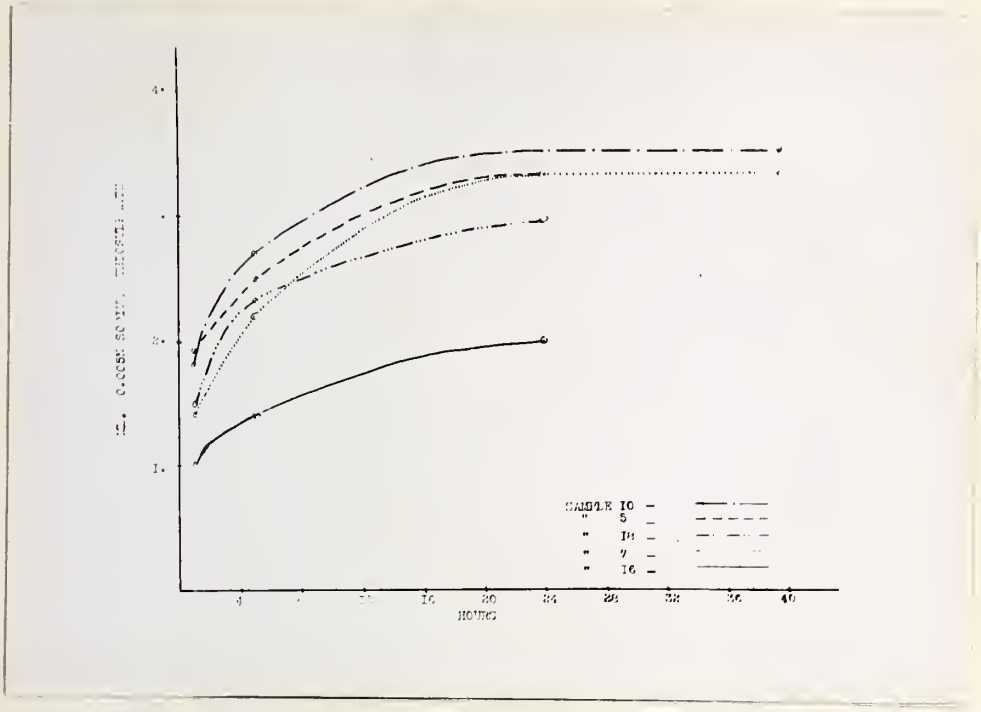

Figure 4. Hydrolysis of 5 typical glycogen samples with Clarase

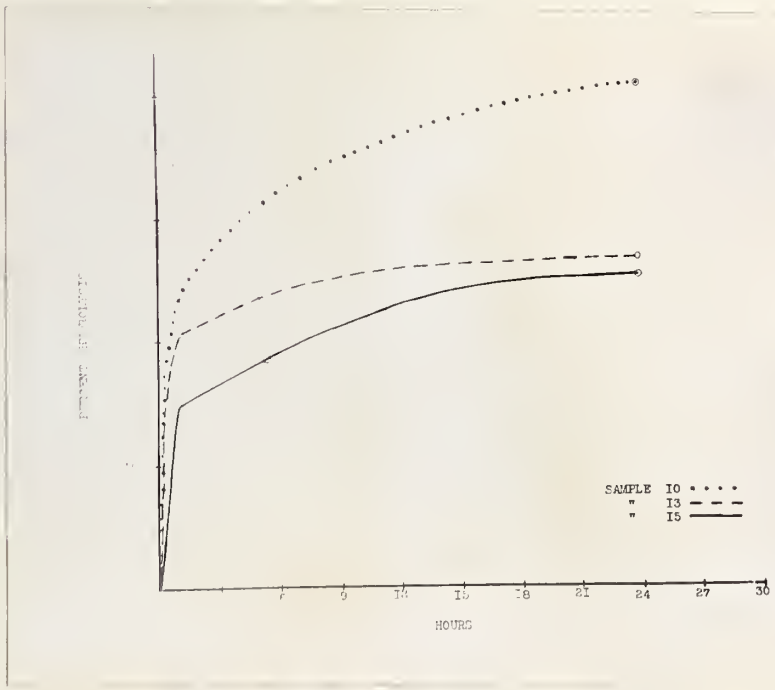

Figure 5. Per cent hydrolysis, by β amylase, of 3 glycogen samples.

TABLE XVll

Estimation of reducing sugar after acid hydrolysis of 1 mgm. glycogen.

Values are reported as (ml. 0.005N $Na_2S_2O_3$)

Sample	Acid Hydrolyzed	Control	% Glycogen
1.....	7.7	0.0	93.0
2.....	7.8	0.0	95.0
3.....	7.8	0.0	95.0
4.....	7.7	0.0	93.0
5.....	7.7	0.0	93.0
6.....	6.2	0.0	75.6
7.....	7.5	0.0	91.4
8.....	7.1	0.0	86.4
9.....	5.7	0.0	69.5
10.....	7.5	0.0	91.5
11.....	5.1	0.0	62.2
12.....	3.3	0.0	40.2
13.....	6.0	0.0	73.2
14.....	4.5	0.0	54.9
15.....	5.5	0.0	67.1
16.....	3.0	0.0	36.5
17.....	5.6	0.0	68.3
18.....	6.5	0.0	79.3

TABLE XVlll

Estimation of reducing sugar after Clarase hydrolysis of 1 mgm. glycogen.

Values are reported as (ml. 0.005N $Na_2S_2O_3$)

Sample	1 hr.	5 hrs.	24 hrs.	48 hrs.
1.....	1.8	2.5	3.4	
2.....	1.7	2.4	3.5	
3.....	1.5	2.1	3.2	
4.....	1.8	2.3	2.7	
5.....	1.9	2.5	3.3	
6.....	1.2	1.6	2.7	
7.....	1.4	2.2	3.3	3.3
8.....	1.3	2.1	2.9	
9.....	0.9	1.3	1.8	
10.....	1.8	2.7	3.5	3.5
11.....	1.0	1.8	2.3	
12.....	0.7	1.2	2.1	
13.....	0.9	1.9	2.0	
14.....	1.0	1.2	1.5	
15.....	1.0	1.4	2.0	
16.....	0.7	1.2	1.6	
17.....	1.2	2.2	2.6	
18.....	1.5	2.3	2.9	
7.....	1.4	2.1	3.2	
10.....	1.7	2.5	3.5	

TABLE XIX

Estimation of reducing sugar after β amylase hydrolysis of 1 mgm. Glycogen.

Values are reported as ml. of 0.005N $Na_2S_2O_3$

ml. 0.005N $Na_2S_2O_3$					% Hydrolysis		
Hours					Hours		
	1	5	24	48	1	5	24
10	0.9	1.2	1.6	1.6	23.6	31.1	41.2
13	0.6	0.7	0.8	0.8	20.7	23.7	26.9
15	0.4	0.5	0.7	0.7	14.8	18.3	25.4

3. Estimation of Protein. The Micro-Kjeldahl method (25) was used to determine the amount of nitrogen in the samples. A conversion factor was used to obtain the per cent protein from the per cent nitrogen. An approximate factor of 6.00 was chosen as this value varies from 5.18 to 6.38 depending upon the source material, whether it be vegetable or animal. It seems reasonable to assume that not all of the nitrogen was in the form of protein.

All the samples were tested in duplicate and some in triplicate. A blank was run with every five samples.

The per cent protein for the samples is shown in the composite table. The values range from a high of 37.68 to a low of 0.84.

The enzymatically prepared samples were higher than the others, except for the redigested sample which was much lower in nitrogen.

4. Estimation of Ash. The ash content of the samples was determined as follows:

Four silica crucibles were brought to constant weight in the muffle furnace at a temperature of approximately 600°C, after which 0.10 grams of glycogen was placed in each crucible and then ashed for one hour. Cooling took place in a desiccator and the ash resulting was calculated to per cent ash. The values are shown in the composite table.

Further proof for the statement made earlier, that the potassium hydroxide treatment requires a ration of one to 3 or less, for protein to potassium hydroxide solution respectively, is obtained when the values for samples 10 and 11 are examined.

The commercial samples showed some variation with samples one and 2 having the highest percentage.

Haworth (22) reported that ash in glycogen samples may be as high as 1.37% but if the sample is highly purified the ash content can be reduced to 0.3 per cent.

3. Coloration of Glycogen with Iodine.

A. Iodine Coloration. Comparison of the iodine coloration of the glycogen samples was made using the Beckman Spectrophotometer.

One ml. of a 0.1% solution of glycogen, which had been centrifuged, was placed in a 50 ml. glass-stoppered volumetric flask to which was added 20 ml. of carbon dioxide-free water. Five ml. of a stock solution of iodine-potassium iodide, was added and the resulting solution was made up to 50 ml. The stock solution contained 2. grams of iodine and 20 grams of potassium iodide in one liter of carbon dioxide free-water. It was prepared at the same time. The solution was left for one hour before the color was examined, using the Beckman Spectrophotometer. The operation of the spectrophotometer was a standard procedure, and will not be outlined here. A measure of the optical density of the blank against carbon dioxide-free water was taken and then the same procedure was carried out for the glycogen solution. Wave lengths ranging from 400 to 800 mμ were used in the determinations with a slit width of 0.06 mm. The resulting value,

when the 2 readings were subtracted, was considered to be due to glycogen plus iodine-potassium iodide. Duplicate readings were made for freshly prepared solutions and the results were nearly identical with the earlier ones.

The enzymatically prepared glycogens gave lower readings at a wave length of 450mμ than did the commercial glycogens and potassium hydroxide and trichloroacetic acid prepared glycogens. The readings obtained are shown in Table XX. The optical density was calculated for 100 per cent glycogen and the results are shown in Table XX. The enzymatically prepared glycogens still have the lowest readings. Plotting the optical density readings against wave lengths for typical samples of each preparation gave curves as shown in Graph number 4, Figure 6.

B. Iodine Coloration after Freezing. In conjuntion with this, a test to determine whether freezing had any effect on glycogen was made by comparing the results with those obtained before freezing. The stock sample of glycogen was frozen for 24 hours and after thawing and centrifuging, one ml. was removed and treated as outlined previously. A new blank was prepared each day. The readings obtained were nearly identical to those of the solution before freezing. These results indicated that freezing had no effect on the solubility of glycogen. The readings obtained using a wave length of 450 mμ are shown in Table XX.

C. Opalescence. A visual appraisal of the opalescence of the 18 stock solutions of glycogen used in this test was made and the

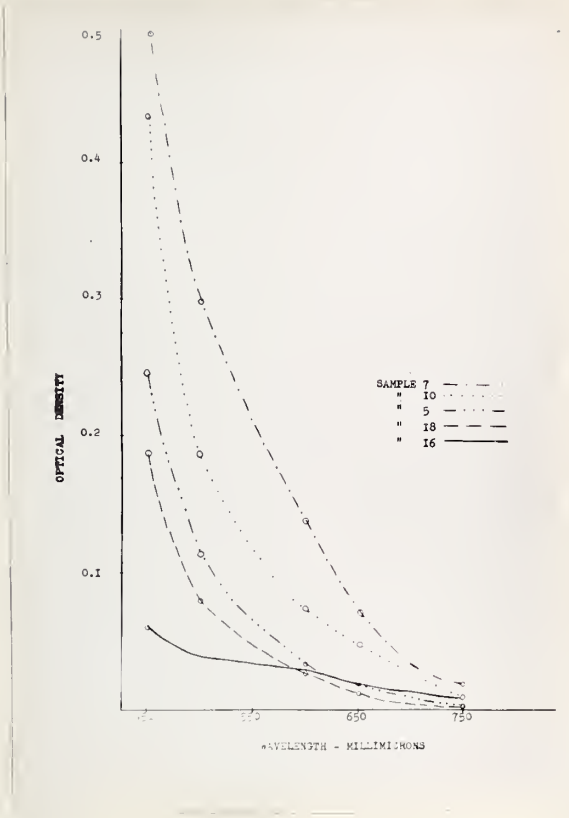

Figure 6. Optical Density, at various wave lengths, of 5 typical glycogen samples.

results are shown in the composite table. Sample number 2 was chosen as the standard. The least opalescence was given by the enzymatically prepared glycogen samples while the most was shown by samples prepared by the potassium hydroxide and trichlorcecetic acid methods using liver as the source material.

TABLE XX

Optical Density at a Wave Length of 450 mμ

	Before Freezing		After Freezing
Sample	100 per cent Purity		
1	0.32	0.34	0.31
2	0.22	0.23	0.20
3	0.30	0.32	0.30
4	0.32	0.34	0.31
5	0.26	0.28	0.25
6	0.19	0.25	0.17
7	0.50	0.55	0.50
8	0.25	0.29	0.24
9	0.10	0.14	0.10
10	0.43	0.47	0.44
11	0.10	0.16	0.10
12	0.08	0.19	0.09
13	0.14	0.19	0.15
14	0.055	0.10	0.051
15	0.11	0.16	0.10
16	0.061	0.17	0.059
17	0.13	0.19	0.13
18	0.19	0.25	0.19

6. Osmotic pressure measurements were made in an attempt to compare the molecular weights of the different samples. After several different osmometers were tested for their operation one conventional type was chosen and is shown in Figure 7. However, this type was still not reliable as there was reason to believe that the osmotic pressure determined by this method was not accurate.

Basis for this observation was the possibility that some of the liquid in the capillary did not take part in the osmotic exchange. A one per cent solution of glycogen in 0.1N calcium chloride was used as the inner liquid while distilled water was used as the outer liquid. The calcium chloride is thought to aid in the dispersal (influences state of hydration) of the glycogen molecule thus lowering the micell formation. A thin cellophane membrane cut from a dialysing bag was used to separate the 2 liquids.

The operation of the osmometer was as follows. Three ml. of the glycogen-calcium chloride solution was placed in the inner chamber (B) with the upper end of the capillary tube clamped off. The cellophane membrane (D) and perforated metal disc (E), which was required to hold the membrane in place, were then inserted. Rubber washer (F) was then placed on top of the metal disc before the cylinder (H) was put into position. Screw cap (I) was then tightened to insure leak-proof joints. The liquid in the capillary was forced to the same level each time so that a constant volume of liquid was in contact with the membrane. The apparatus was completely immersed in a battery jar (K) containing distilled water at a temperature of 30°C. This was then placed in the incubator and maintained at 30°C.

The liquid ceased rising after approximately 6 hours in all cases and after one hour the level of the liquid in the capillary tube gradually fell. This cessation of rise of the liquid was not due to a perforated membrane as the same membrane could be used with a duplicate solution to give the same reading. The possibility that calcium chloride might alter the pore size of the cellophane

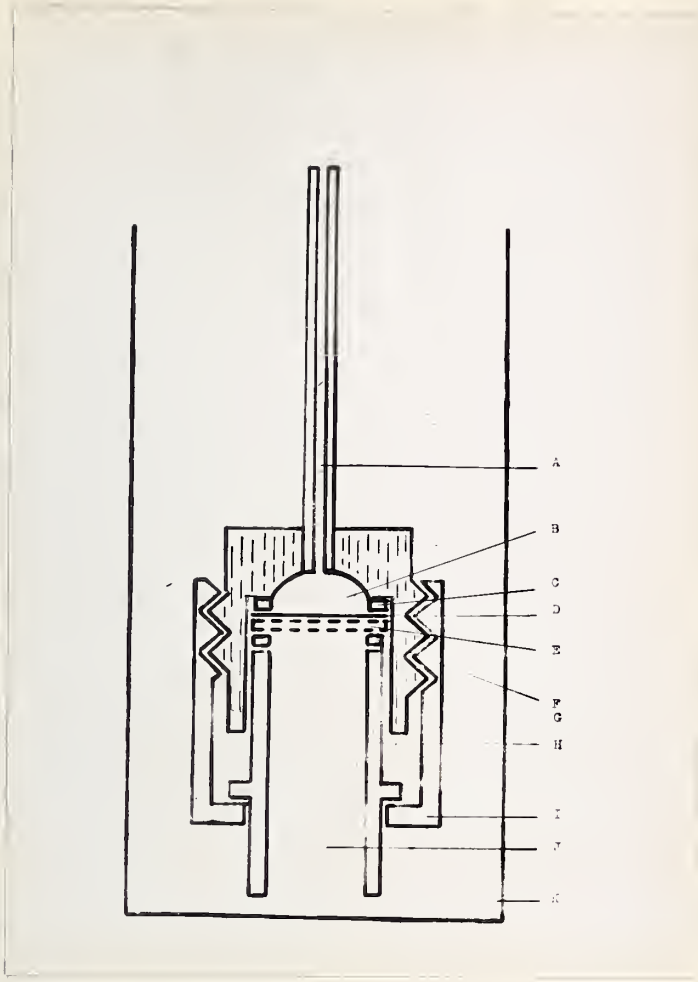

Figure 7. Osmotic pressure apparatus.

A. Capillary tube.
B. Inner chamber.
C. Rubber washer.
D. Cellophane membrane.
E. Perforated metal disc.
F. Rubber washer.
G. Metal container.
H. Cylinder.
I. Screw cap.
J. Outer chamber.
K. Battery jar.

membrane was tested. It was noted that after the membrane had been soaked in the calcium chloride solution for 6 hours and then immediately used for osmotic pressure testing, that the glycogen solution still had a capillary rise for 6 hours.

Results of the test are shown in the composite table. Although the accuracy of the results was not great, variations were observed between the samples.

Molecular weight determinations were made using the equation:

M.wt. = 10.RT.C/P at 25°C reported by Oakley and Young (31). No value over 240,000 was found but it is of interest to note that the 2 highest values were obtained with samples (1) and (2) which had the highest per cent glycogen based on acid hydrolysis. One of the samples (1) that had a high purity also had a very low molecular weight.

7. Viscosity. Viscosity tests were made using the Ostwald viscosimeter #100.

Five ml. of a 0.5% solution of glycogen using distilled water as the solvent was added to the viscosimeter which was then placed in a constant temperature bath set at 30°C. The solution of glycogen was centrifuged before being placed in the viscosometer. After 20 minutes had elapsed, to allow the solution and apparatus to reach the temperature of 30°C, 10 readings were taken (using a stop watch). A sample of distilled water was tested in the same manner.

The results in the composite table show a range of results from 49.0 seconds to 53.8 seconds with an average time of 50.8 seconds. The time in seconds was converted to η (poise) for each sample.

Two of the enzymatically prepared samples and one of the potassium hydroxide prepared samples gave the highest values. The picric acid method was the only one which did not produce a sample with a low value.

8. Periodate Oxidation.

The use of periodate oxidation for end group analysis of polysaccharides has been mentioned frequently in the literature. This procedure was employed on the glycogen samples after careful study of the procedures used by various workers (37, 19). Periodic acid was used in place of sodium metaperiodate and the procedure was as follows.

The stock solution of periodic acid was prepared as follows:

Seven grams of periodic acid was dissolved in 30 ml. of carbon dioxide-free water. This solution was brought to a pH of 4.2 with one N sodium hydroxide. The volume was then made up to 100 ml. before it was used.

One-tenth of a gram of glycogen was placed in a 50 ml. glass-stoppered volumetric flask to which was added 30 ml. of carbon dioxide free-water. Ten ml. of a freshly prepared stock solution of periodic acid was added and the volume was made up to 50 ml. with carbon dioxide free-water. This solution was incubated at 30°C for 24 hours and 5 ml. portions were taken at the end of 1, 5 and 24 hours. A blank was run in conjunction with this test. Six drops of ethylene glycol was added to the 5 ml. portion to destroy the periodic acid present. After 10 minutes the solution was titrated with 0.01N sodium hydroxide (carbonate free) using methyl red as an indicator. The number of ml. of 0.01N sodium

hydroxide required are recorded in Table XXI. Graph number 5 (Figure 8) shows the curves of the typical samples representing each type of glycogen. After 24 hours it is believed that secondary oxidation takes place, thus the values would not be valid.

Chain lengths for the individual samples were calculated from the values using the formula given by Potter and Hassid (37).

$$\text{Chain length} = 0.283 \times \frac{\text{gm. glycogen}}{\text{gm. Formic acid.}}$$

The chain length values in some cases were very high and in others very low. The samples that were high in glycogen content had short chain lengths while the samples that had a low purity had long chain lengths. All the enzymatically prepared samples had a high chain length value while all the others, with the exception of one potassium hydroxide prepared sample, had a low value. These values are shown in Table XXI.

Two different concentrations of bovine albumin and a so-called yeast protein were substituted for glycogen in the above reaction. Both reaction mixtures became acidic with a higher acidity for the yeast protein than for the bovine albumin. A reducing sugar determination revealed that the yeast protein contained the equivalent of 52.4 per cent glycogen while the bovine albumin contained no carbohydrate. The carbohydrate present in the yeast protein was not necessarily glycogen. This yeast protein was not the same source material as that used in the preparation of the glycogen samples studied.

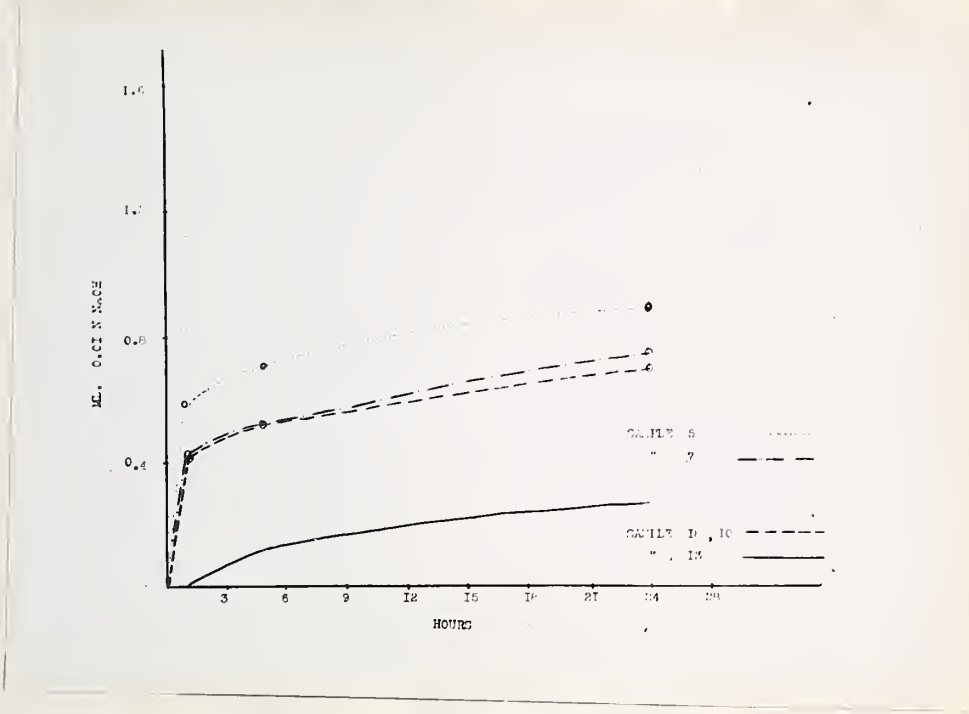

Figure 8. Periodic Acid Oxidation of 5 typical glycogen samples.

If a correction was made for this yeast protein effect for the samples of glycogen prepared from yeast, the chain lengths were found to be greater. Similar results were obtained if a correction was made for bovine albumin, although it is not known whether albumin or some other protein is the protein present with glycogen.

Not too much value is attached to these individual chain lengths as there appears to be a great deal of controversy in the literature with respect to the action of periodic acid on polysaccharides.

TABLE XXI

ml. of 0.01N NaOH required in the periodic acid oxidation reaction over a period of 24 hours.

Sample	1 hr.	5 hr.	24 hr.	Chain Length
1	0.42	0.53	0.69	8.9
2	0.58	0.77	0.96	6.5
3	0.47	0.57	0.72	8.6
4	0.41	0.50	0.64	9.8
5	0.58	0.71	0.90	6.9
6	0.49	0.60	0.78	8.1
7	0.42	0.53	0.76	8.4
8	0.33	0.58	0.81	7.7
9	0.10	0.30	0.58	10.9
10	0.41	0.53	0.71	8.9
11	0.12	0.34	0.65	10.3
12	0.23	0.29	0.39	15.8
13	0.00	0.11	0.29	21.9
14	0.00	0.05	0.27	23.7
15	0.00	0.12	0.32	18.9
16	0.00	0.00	0.15	05.7
17	0.23	0.40	0.49	12.3
18	0.42	0.53	0.71	8.6

9. Cupric Crystallization Pattern

Morris and Morris (28), in their report on glycogen found in sweet corn, describe a method whereby a solution of cupric chloride

crystallizes into a definite pattern when it contains glycogen or amylopectin and amylose. Their method is outlined below.

Three identical watch glasses were cleaned and to one, 19 mg. of glycogen was added, to another 19 mg. of amylopectin, and to the remaining one a solution of amylose containing the desired amount. To each watch glass was added 8 ml., or the equivalent of 6.25 per cent cupric chloride solution, and after the solid material had saturated the solution, the 3 watch glasses were placed in a desiccator. Three different crystalline patterns resulted after the solutions were evaporated to dryness. A photograph of the resulting patterns is shown in Figure 9. A visible difference between glycogen and amylopectin was quite marked but not so between glycogen and amylose.

The test is not of great importance but does illustrate that the molecular structure does have some effect on the crystallization of cupric chloride.

10. Protein removal is the main problem in the preparation of glycogen. Several tests were carried out in an effort to determine whether glycogen is bound to protein in vitro.

A 0.2% solution of commercial glycogen and a one % solution of egg albumin were tested separately for their reducing sugar value. The results are shown in Table XXII.

Twenty-five ml. of a 0.2% solution of glycogen was mixed with 25 ml. of a one per cent solution of egg albumin and the mixture was heated for 2 hours in boiling water at a pH of 6.5. An aliquot of the mixture was tested for reducing sugar after this period of heating. The protein was then precipitated by the addition of 5 ml. of sodium

acetate-acetic acid buffer (pH. 4.67, 0.1M) and then an aliquot of the glycogen solution was tested.

The values in Table XXII indicate that very little, if any, of the glycogen was bound to or removed with the albumin and that the acid hydrolysis of the glycogen was not affected.

The same procedure as stated above was carried out at a temperature of 37°C for 24 hours using toluene as a preservative. An aliquot of this mixture was also tested for reducing sugar. The protein was then removed by heating to 80°C in the presence of 5 ml. of sodium acetate-acetic acid buffer and an aliquot of the glycogen solution was tested.

The values given in Table XXII for reducing sugar again indicate that little, if any, glycogen was bound to the albumin.

A mixture of 25 ml. of one % glycogen and 25 ml. of one % egg albumin was heated for 2 hours at a pH of 6.5. One-tenth the volume of 40% trichloroacetic acid was added and the resulting suspension and subsequent precipitation of the supernatant with 2.3 volumes of 95% ethyl alcohol completed the procedure. The sample was washed and dried as previously outlined. The yield equalled 0.2051 grams and the reducing sugar test valuewas 7.4.

From a study of the table and the above procedures it was possible to postulate that glycogen and albumin have no significent amount of binding in vitro. Further, albumin itself interfers only slightly in the reducing sugar test used in this study.

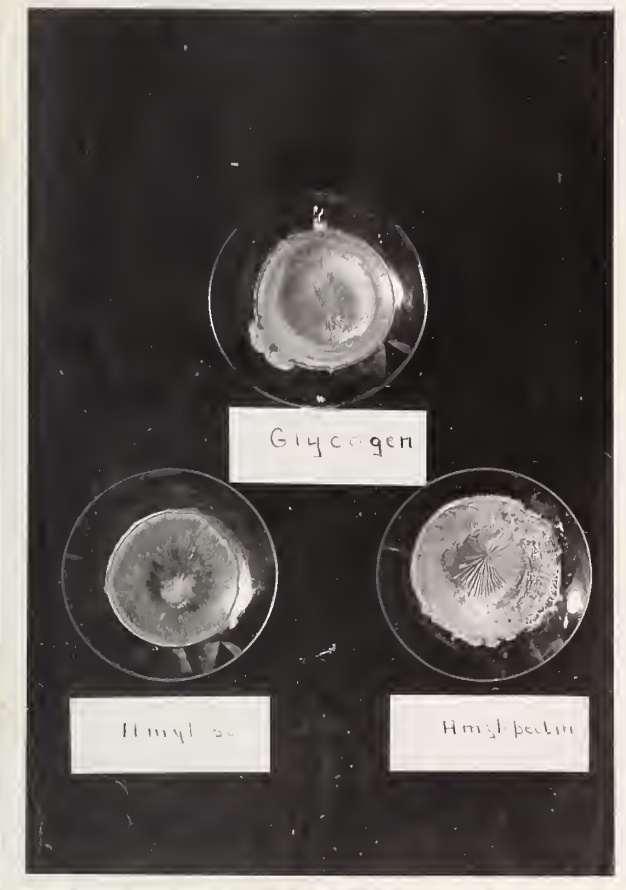

Figure 9. Cupric chloride crystallization patterns for glycogen, amylopectin and amylase.

TABLE XXII

Estimation of reducing sugar after acid hydrolysis of various solutions.

Values are reported as (ml. 0.005N $Na_2S_2O_3$)

Solution	Acid Hydrolyzed	Control
Albumin 1 mg.	0.4	0.3
Glycogen 1 mg.	7.7	0.0
Glycogen plus Albumin Mixture after 2 hrs. heating.	7.6	0.3
Glycogen solution after the Albumin was removed.	7.6	0.1
Glycogen plus Albumin Mixture after incubation for 24 hrs.	7.7	0.3
Glycogen solution after the albumin was removed.	7.6	0.2
Precipitated Glycogen.	7.4	0.1

11. Iron Test. A solution of each of the 18 glycogen samples was tested for the presence of iron, using potassium thiocyanate as the reagent. None of the samples gave a positive test, thus it was concluded that iron (Fe^{+++}) was not present.

DISCUSSION

The evaluation of each of the methods used to prepare glycogen was presented at the end of the description of each modification. The yields are summarized in Table XXIII. The yields represent the weights of the final dried samples as percentages of the original fresh source materials; the yield purity values represent the yields of pure glycogen. In preparations where several precipitations were made, the average purity was used for the calculation of the yield purity value. Only one of the yields based on purity exceeded 1.0 per cent. The exception was a sample, prepared from calf liver by the potassium hydroxide method, which gave a yield of 1.69 per cent. Certain low yields may be attributed to the drastic modification of the common method.

Using 3 portions of a single liver it was found that the potassium hydroxide method gave a yield of 1.69 % based on purity while the trichloroacetic acid method gave a 0.54 % yield and the enzymatic 0.27 %. This indicates that the potassium hydroxide method extracts more glycogen and glycogen of a higher purity than the others. This confirms the finding of Bloom et al (9) who reported that trichloroacetic acid extracts, less glycogen than potassium hydroxide.

The values in Table XXIII indicated that oysters and calf liver are better source materials than yeast. The highest yield, using yeast, is 0.41 % while oysters yielded 1.12 % and liver, the highest, 1.69 %.

The highest value obtained for ash was 59.88 %, found in a sample prepared by the potassium hydroxide method. The lowest ash was 0.50 %, found in a glycogen prepared by the trichloroacetic acid method. The trichloroacetic acid method was the only one that produced consistently low ash values.

All of the glycogen samples prepared enzymatically showed a high ash even after continuous dialysis for 2 days. It is possible that the ash, as in amylopectin, is bound to the glycogen molecule, thus accounting for its presence after dialysis. No analysis of the ash was made.

It was not surprising to find that the hot concentrated potassium hydroxide method produced the purest sample when it was considered that such treatment causes extensive hydrolysis of protein. The trichloroacetic acid method removes less protein, possibly due to its different mode of action and to the low temperature used. There was 6 times as much protein in the sample of glycogen using this method as in the sample obtained using potassium hydroxide on the same source material. The enzymatically prepared samples contained a high percentage of protein, even in the purest preparations.

All the methods produced samples containing some protein which might indicate that glycogen is bound in some way to protein in vivo. Tests made to determine whether glycogen and protein (egg albumin) show any binding in vitro revealed that they did not.

αAmylase action revealed that pure commercial glycogens and pure samples prepared using the potassium hydroxide and trichloroacetic

acid methods, have either relatively few long chains or a greater number of short chains. This statement is based on the continued high rate of hydrolysis after 5 hours which was not observed with the less pure glycogen samples. There is, however, a possibility that the amylase action is faster in the less pure glycogens because there is a higher proportion of enzyme to substrate.

β Amylase action revealed that glycogen prepared using potassium hydroxide had more chains or longer ones than the enzymatically prepared glycogens. With the former, 41.2 % hydrolysis occurred while with the latter only 25.4 % to 26.2 % hydrolysis occurred.

The periodic acid oxidation study was not considered reliable for chain length determinations because yeast protein and albumin were found to cause the production of some acid. The values obtained indicated that low purity glycogen had chain lengths from 15 to 35 while the purer glycogens had chain lengths of 7 to 12. The chain length of glycogen is reported to be in a range from 10 to 23 glucose units but consideration of interference from protein has not been mentioned.

Molecular weights were calculated and found to range from 42,000 to 240,000, which is in agreement with the reported molecular weights of glycogen. These values were not considered to be too reliable because the osmometer used was not satisfactory.

No relationship between viscosity and purity was observed and, although the values could be duplicated, they were not considered to be very useful.

Iodine coloration measurements were closely related to purity. This relationship did not change after freezing a glycogen solution, indicating that glycogen is soluble after freezing. Amylopectin, a related branched polysaccharide, is not soluble after freezing. This characteristic is believe to be due to the longer chains present in amylopectin which, like those of amylose, are degraded when subjected to abnormal conditions. The coloration of glycogen is believe to depend upon the iodine reaction with the chains; thus, the longer the chain the more intense the coloration. It is believed that the coloration with iodine and glycogen begins to intensify when the chains contain 8 or more glucose units.

TABLE XXIII

Relationship of per cent yield of pure glycogen to the source and method.

Source	Method	% Total Yield	% Yield Purity
Yeast	Enzymatic #3	1.12	0.41
Yeast	" #4	0.67	0.31
Liver	" #6	1.48	0.27
Liver	" #7	0.60	0.20
Oysters	" #8	1.36	0.74
Oysters	" #9	0.17	0.12
Yeast	KOH #1	0.49	0.35
Liver	" #2	1.85	1.69
Liver	" #3	0.27	0.17
Liver	TCA #1	0.62	0.54
Liver	" #2	0.31	0.28
Oysters	" #3	1.49	1.12
Oysters	Picric acid (Petree)	1.13	0.77
Oysters	" (Meyer)	1.41	1.12

SUMMARY

1. Various methods for the preparation of glycogen have been tested and the products have been analysed and various properties examined.

2. An enzymatic method for preparing glycogen was developed and the products compared with those of the other methods.

3. The potassium hydroxide and trichloroacetic acid methods produced glycogen samples which were both 91.5 % pure. These samples were prepared from calf liver.

4. The picric acid method, described by Meyer, produced a sample of higher purity than the picric acid method, described by Petree and Alsberg, using oysters as the source material. The per cent purity of the former was 79.3 and of the latter, 68.3.

5. The sample prepared enzymatically contained 73.2 % glycogen. This sample was prepared from fresh brewer's yeast.

6. The trichloroacetic acid method was the simplest to carry out but the yield was lower than that obtained from the next in order of preference, the potassium hydroxide method.

7. Neither of the picric acid methods removed protein successfully which, together with the expense of the procedure, made them undesirable.

8. The enzymatic method was very time-consuming. It was most effective on brewer's yeast and least effective on calf liver. Hemicellulase was used to aid in the breakdown of the wall of the yeast cell but was not satisfactory due to the amylase activity of hemicellulase.

9. The feeding of freshly collected brewer's yeast with a 5 % sucrose solution, before extraction of the glycogen, did not increase the yield.

10. Neutralization of the alkaline solution in the potassium hydroxide method, before the addition of 95 % ethanol, did not improve the method.

11. Fractional precipitation of the glycogen containing solution to remove undesirable materials was not found to be useful. The addition of 2.5 volumes of 95 % ethanol was found to be satisfactory for the precipitation of glycogen from the solution.

12. The ratio of one part of source material to 3 parts of potassium hydroxide solution was found to be more satisfactory than a ratio of 1:2 or 1:1.

13. The commercial samples had a purity ranging from 93 % to 95 % with a range of 0.84 to 2.10 for per cent protein. The per cent ash ranged from 0.30 to 0.61.

14. α Amylase hydrolysis of the glycogen samples was found to correspond to the purity of the sample and showed a curve typical for enzymatic action. The α amylase action went to completion more rapidly with the low purity samples than it did with the high purity samples.

15. β Amylase action was found to hydrolyse 41.2% of the glycogen in a sample of high purity while only 26.9 % hydrolysis occurred in a sample of low purity.

16. Coloration of the glycogen with iodine was closely related to the purity of the sample.

17. No change in the iodine coloration was noted after a glycogen sample had been frozen and then thawed.

18. The trichloroacetic acid and potassium hydroxide methods produced glycogen that gave a more opalescent solution than the glycogens resulting from the other methods.

19. Viscosity measurements did not reveal any well defined relationship to purity.

20. Molecular weights ranging from a high of 240,000 to a low of 92,000 were obtained using osmotic pressure measurements.

21. Protein and glycogen when mixed in vitro did not show any binding.

22. There is reason to believe that protein impurity in a glycogen sample may influence the periodic acid oxidation reaction and thus the calculated chain length for the glycogen sample concerned.

23. Cupric chloride crystallization patterns were found to distinguish between glycogen and amylopectin.

24. The presence of iron in the ferric form was not found.

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COMPOSITE TABLE

	SAMPLE OF GLYCOGEN	% Moisture	% Protein	% Ash	Total % Protein, & Ash.	Total % Protein Glycogen & Ash.	A ml. Na_2S_2
		Max. Min.	Max. Min.	Max. Min.			
1.	Commercial glycogen.....oysters)	11.03 +	1.92	0.40 -	2.32	95.32	7.7
2.	Pfanstiehl glycogen.....C.P.) Commercial preparations	6.65	2.10	0.61	2.71	97.71	7.8
3.	Pfanstiehl glycogen.....practical) of glycogen.	12.11 +	0.84 -	0.60	1.44	96.44	7.8
4.	General Biochemicals.....)	7.32	1.26	0.30 -	1.56	94.56	7.7
5.	Nutritional Biochemicals)	7.33	0.84 -	0.50	1.34	94.34	7.7
6.	Trichloroacetic glycogen.....oysters, preparation 3.	6.43 -	16.80	0.50	17.30	92.90	6.2
7.	Trichloroacetic glycogen.....calf liver, preparation 2.	7.77	6.30	0.88	7.18	98.58	7.5
8.	Trichloroacetic glycogen.....calf liver, preparation 1.	8.06	12.30	0.79	13.09	99.49	7.1
9.	Potassium hydroxide glycogen.....yeast, preparation 1A.	13.70 +	13.86	0.30 -	14.16	83.56	5.7
10.	Potassium hydroxide glycogen.....liver, preparation 3.	8.69	2.16	6.71	8.87	100.37	7.5
11.	Potassium hydroxide glycogen.....liver, preparation 4.	9.73	25.20 +	7.81	33.01	95.20	5.1
12.	Potassium hydroxide glycogen.....liver extract, preparation 4.	3.25 -	0.84 -	59.88 +	60.72	100.92	3.3
13.	Enzymatic glycogen.....yeast (protein plus trypsin plus papain, second precipitate), preparation 3.	7.54	19.20 +	4.31	23.51	96.71	6.0
14.	Enzymatic glycogen.....yeast redigestion, preparation 3.	4.09 -	9.72				4.5
15.	Enzymatic glycogen.....oyster, preparation 9.	7.77	18.84	11.38 +	30.22	97.32	5.5
16.	Enzymatic glycogen.....liver extract, preparation 10.	12.83	37.68 +	25.22 +	62.90	99.40	3.0
17.	Picric acid glycogen (Petree and Alsberg).....oysters, preparation 1.	7.65	3.30	2.91	6.21	74.54	5.6
18.	Picric acid glycogen (Meyer).....oysters, preparation 1.	8.77	1.44	0.70	2.14	81.44	6.5
	Water						
	Bovine Albumin						
	Calcium Chloride						

Total % Protein Glycogen & Ash.	Acid Hydrolysis ml. $Na_2S_2O_3$	% Purity			Clarase ml. of 0.005N $Na_2S_2O_3$ Hours		Hydrolysis 0.005N $Na_2S_2O_3$				Amylase Hydrolysis ml. of 0.005N $Na_2S_2O_3$ Hours						% Hydro-lysis	Viscosity Measurements Seconds	Centipoise			Osmometric Measurements mm. of H_2O	Mol. Wt.			Fer m Hours	
			Max.	Min.			24	48	Max.	Min.	1	5	24	48	Max.	Min.				Max.	Min.			Max.	Min.	1	5
95.32	7.7	93.0	+		1	1.8	2.5	3.4		+								50.2	0.8271			10.5	240,000	+		0.42	0.53
97.71	7.8	95.0	+		2	1.7	2.4	3.5		+								50.3	0.8287			11.0	230,000	+		0.58	0.77
96.44	7.8	95.0	+		3	1.5	2.1	3.2										50.9	0.8386			17.0	150,000			0.47	0.57
94.56	7.7	93.0	+		4	1.8	2.3	2.7										50.1	0.8254			17.5	140,000			0.41	0.50
94.34	7.7	93.0	+		5	1.9	2.5	3.3										50.1	0.8254			21.5	120,000			0.58	0.71
92.90	6.2	75.6			6	1.2	1.6	2.7										49.0	0.8073		-	12.5	200,0000	+		0.49	0.60
98.58	7.5	91.4	+		7	1.4	2.2	3.3	3.3									51.7	0.8518			14.5	170,000			0.42	0.53
99.49	7.1	86.4			8	1.3	2.1	2.9										50.4	0.8304			23.0	100,000			0.33	0.58
83.56	5.7	69.5			9	0.9	1.3	1.8		-								53.8	0.8863	+		21.0	120,000			0.10	0.30
100.37	7.5	91.5	+		10	1.8	2.7	3.5	3.5	+	0.9	1.2	1.6	1.6	+		41.2	51.8	0.8534			51.0	50,000		-	0.41	0.53
95.20	5.1	62.2			11	1.0	1.8	2.3										49.6	0.8173		-	24.5	100,000			0.12	0.34
100.92	3.3	40.2		-	12	0.7	1.2	2.1										50.1	0.8254			59.5	40,000		-	0.23	0.29
96.71	6.0	73.2			13	0.9	1.9	2.0			0.6	0.7	0.8	0.8			26.9	50.6	0.8337			48.0	50,000		-	0.00	0.11
	4.5	54.9		-	14	1.0	1.2	1.5		-								52.3	0.8617	+		43.5	60,000			0.00	0.05
97.32	5.5	67.1			15	1.0	1.4	2.0			0.4	0.5	0.7	0.7		-	25.4	53.1	0.8748	+		24.5	100,000			0.00	0.12
98.40	3.0	36.5		-	16	0.7	1.2	1.6		-								49.8	0.8204		-	32.5	77,000			0.00	0.00
74.51	5.6	68.3			17	1.2	2.2	2.6										50.4	0.8304			24.0	100,000			0.23	0.40
81.44	6.5	79.3			18	1.5	2.3	2.9										50.3	0.8287			37.5	67,000			0.42	0.53
																		48.6	0.8007								
																		59.1	0.9737			16.5	150,000				
																						4.85					

Periodate Oxidation ml. of 0.01 NaOH Hours 1	5	24	Chain Length	Max.	Min.	Before freezing Optical Density 750	650	600	500	450	Max.	Min.	Iodine Coloration 100 % Glycogen 450	750	After freezing Optical Density 650	600	500	450	Max.	Min.	Opalescence Visual Observation		Iodine Coloration Visual Observation	Sample Color
0.42	0.53	0.69	8.9			0.002	0.019	0.039	0.149	0.32	+		0.34	0.002	0.018	0.039	0.135	0.31	+		1+	1	1	white
0.58	0.77	0.96	6.5	-		0.006	0.025	0.042	0.114	0.22			0.23	0.006	0.021	0.039	0.110	0.20			1(standard)	2	1 (red)	white
0.47	0.57	0.72	8.6			0.002	0.014	0.035	0.140	0.30			0.32	0.003	0.016	0.040	0.144	0.30			1	3	1	white
0.41	0.50	0.64	9.8			0.008	0.030	0.056	0.164	0.32	+		0.34	0.009	0.031	0.054	0.156	0.31	+		1	4	1	white
0.58	0.71	0.90	6.9	-		0.005	0.050	0.040	0.122	0.26			0.28	0.005	0.020	0.034	0.115	0.25			1	5	1	white
0.49	0.60	0.78	8.1			0.011	0.031	0.042	0.096	0.19			0.25	0.010	0.029	0.041	0.085	0.17			1	6	1-	white
0.42	0.53	0.76	8.4			0.021	0.081	0.142	0.305	0.50	+		0.55	0.019	0.072	0.141	0.303	0.50	+		1+	7	1	white
0.33	0.58	0.81	7.7	-		0.015	0.052	0.067	0.151	0.25			0.29	0.015	0.056	0.060	0.160	0.24			1	8	1	white
0.10	0.30	0.58	10.9			0.004	0.017	0.026	0.059	0.10			0.14	0.004	0.015	0.026	0.046	0.10			1-	9	1-	cream
0.41	0.53	0.71	8.9			0.008	0.048	0.076	0.189	0.43	+		0.47	0.008	0.048	0.077	0.191	0.44			1+	10	1+	white
0.12	0.34	0.65	10.3			0.005	0.020	0.030	0.057	0.10			0.16	0.006	0.018	0.031	0.057	0.10			1-	11	1--	tan
0.23	0.29	0.39	15.8			0.005	0.017	0.030	0.061	0.08		-	0.19	0.003	0.019	0.028	0.060	0.09		-	1-	12	1-	light grey
0.00	0.11	0.29	21.9	+		0.008	0.023	0.041	0.080	0.14			0.19	0.007	0.024	0.037	0.079	0.15			1--	13	1--	tan
0.00	0.05	0.27	23.7	+		0.005	0.017	0.027	0.035	0.055		-	0.10	0.004	0.019	0.026	0.033	0.051		-	1---	14	1---	cream
0.00	0.12	0.32	18.9			0.015	0.036	0.052	0.073	0.11			0.16	0.014	0.030	0.052	0.072	0.10			1---	15	1--	white
0.00	0.00	0.15	35.7	+		0.009	0.022	0.032	0.039	0.061		-	0.17	0.009	0.020	0.033	0.037	0.059		-	1---	16	1---	tan
0.23	0.40	0.49	12.3			0.004	0.016	0.036	0.079	0.13			0.19	0.003	0.017	0.031	0.081	0.13			1--	17	1-	tan
0.42	0.53	0.71	8.6			0.004	0.018	0.038	0.083	0.19			0.25	0.006	0.015	0.027	0.079	0.19			1-	18	1-	white